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# PATHOGENIC MICROÖRGANISMS

A PRACTICAL MANUAL FOR STUDENTS, PHYSICIANS  
AND HEALTH OFFICERS

BY

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## PREFACE TO THE SIXTH EDITION.

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THE first edition of this book was called *Bacteriology in Medicine and Surgery*. It was written to make available for others the practical knowledge which had been acquired in the work of the bacteriological laboratories of the city of New York, and was intended more for medical practitioners than for medical students or laboratory workers. When the second edition had been exhausted the improvement in methods of cultivating and studying the protozoa had reached a point rendering it advantageous to include the animal as well as the vegetable germs. This was done and the title of the third edition was altered to conform with the text, which had been broadened to give in outline practically the whole field of pathogenic microorganisms.

In the fifth edition the material was rearranged in order to bring more closely together all of the pathogenic organisms. Under this arrangement Part I deals with the general characteristics and methods of study of all the microorganisms considered (molds, yeasts, bacteria, and protozoa). Part II includes the study of the individual pathogenic microorganisms and their near relatives. Part III presents certain practical aspects of the subject under the title Applied Microbiology. In the present edition the practical application of serums and vaccines has been transferred also to Part III. The nine plates included in this edition have been arranged and drawn especially for this book.

Owing to recent advances in the knowledge of microbiology we have added much new material and rewritten several parts of the book. The whole subject of immunity has been extensively revised. A number of recent media and stains have been added in their respective sections. The chapter on Intestinal Bacteria has been rewritten. The experience gained in the war with preventive measures against typhoid, paratyphoid, tetanus, and wound infections has been added. All of the anaërobes are brought together in one chapter and revised. The use of tuberculins in diagnosis and treatment has been revised by Dr. G. B. White. The chapter on Glanders was again revised by Dr. B. Van H. Anthony. The chapter on Complement-fixation was revised by M. A. Wilson, and that on Disinfection by W. C. Noble. The section on Ferments and Antiferments was written by Dr. R. L. Kahn. The

bibliography has been revised, with special reference to the needs of the student rather than to the complete recognition of contributions of investigators. The index and much of the proof-reading was done by C. Van Winkle. We are further indebted to our associates in the laboratory for aid in many different ways.

Dr. Krumwiede's name has been added to the title page because of the valuable assistance he has given us.

W. H. P.

A. W. W.

NEW YORK, 1917.

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# PATHOGENIC MICROÖRGANISMS.

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## PART I.

### PRINCIPLES OF MICROBIOLOGY.<sup>1</sup>

---

#### CHAPTER I.

##### INTRODUCTORY HISTORICAL SKETCH.

ALTHOUGH most of the more important discoveries in microbiology which place it on the footing of a science are of comparatively recent date, the foundations of its study were laid over two centuries ago. From that time the history of microörganisms has been intimately associated with that of medicine. Indeed, it is only through the investigations into the life history of these minute forms that our present knowledge of the etiology, course, and prevention of the infectious diseases has been acquired. The prominent position which the study of microörganisms already holds toward medicine is, moreover, daily increasing in importance. Original discoveries are constantly adding to our knowledge of germ diseases, and the outlook is favorable for eventually obtaining, through serums, through attenuated cultures, or through the toxic substances produced by microörganisms themselves, means for immunizing against, if not of curing, an increased number of the specific infections. Even at present, bacterial products and protective serums are used successfully as preventive or curative agents in several of the most prevalent infectious diseases. Our knowledge concerning other microörganisms has enabled us largely to limit their dissemination and so to prevent disease. An acquaintance, therefore, with the main facts concerning these microörganisms is most necessary to the education of the modern physician.

The vast majority of the known microörganisms which cause disease belong to the closely related groups of lowest plants and animals, *i. e.*, the bacteria, the molds, the yeasts, and the protozoa. A few of

<sup>1</sup> The correct form of this word is under discussion. According to derivation—*microbe* + *logia*—the better spelling would be *microbiology*, but the form given above is the one accepted at present in the dictionaries.

the pathogenic metazoa (some of the parasitic worms) in some of their stages are also microscopic; therefore microscopic methods of study are also applicable to them; but since they are fully presented in works on clinical microscopy they are not given here.

Before entering into a detailed consideration of the subject it may be interesting and instructive to review very briefly a few of the important steps which led to the development of the science, and upon which its foundation rests, in which we shall see that the results obtained were gained only through long and laborious research and after many obstacles were met and overcome by accurate observation and experiment.

Probably the first authentic observations of living microscopic organisms of which there is any record are those of Kircher, in 1659. This original investigator demonstrated the presence in putrid meat, milk, vinegar, cheese, etc., of "minute living worms," but did not describe their form or character.

Not long after this, in 1675, Leeuwenhoek observed in rain-water, putrid infusions, and in his own and other saliva and diarrheal evacuations living, motile "animalcula" of most minute dimensions, which he described and illustrated by drawings. Leeuwenhoek practised the art of lens grinding, in which he eventually became so proficient that he perfected a lens superior to any magnifying glass obtainable at that day, and with which he was enabled to see objects very much smaller than had ever been seen before. "With the greatest astonishment," he writes, "I observed distributed everywhere through the material which I was examining animalcules of the most microscopic size, which moved themselves about very energetically." The work of this observer is conspicuous for its purely objective character and absence of speculation; and his descriptions and illustrations are done with remarkable clearness and accuracy, considering the imperfect optical instruments at his command. It was not until many years later, however, that any attempt was made to define the characters of these minute organisms and to classify them systematically.

At that time all of the microscopic organisms seen were classed together as little animals. Indeed, all of the microorganisms first described at any length were probably protozoa, and only after further improvement of lenses and a more minute study of the organisms were bacterial forms gradually recognized as a separate class.

The same skepticism that is seen in the acceptance of most new discoveries was displayed by doubters of the truth of these early reports of microscopic findings. Chief among the skeptics must be placed Linnaeus, who in the first edition of his *Systema Naturæ* (1735) absolutely denies the existence of Leeuwenhoek's animalcula, though in the later editions he grudgingly admits them under the significant generic name of *Chaos* (*Chaos proteus* (*Ameba*), etc.).

The first ideas of the structure of the protozoa were drawn from analogy. The early observers thought that each tiny organism possessed an internal structure made up of organs and tissues similar

to those in metazoa. They could not conceive of motion without articulation, tendons, and muscles; nor of food absorption without an alimentary tract, and they were so impressed with the ideas of what they thought they ought to see that they were convinced that they really saw many of the complicated structures possessed by metazoa. For example, the contractile vacuole, a characteristic pulsating vesicle of the protozoa, discovered by Joblot in 1754, was thought by many to be lungs, other vacuoles were said to be stomachs, the mouths were often seen and the rest of the alimentary tract was supplied from the imagination; the red pigment spots of many forms were interpreted as true eyes, etc. There were many opponents to these views, however, and the idea of the cell being the unit of structure, which was advanced by Schleiden in 1838, helped determine the fact that protozoa were single cells with no definite organic structure.

With the publications of Dujardin (1835-41) a correct idea of the structural simplicity of the microorganisms gained ground. But for some time after, the controversy regarding the simple nature of protozoa was strenuously carried on. It is a most instructive bit of history in research work, showing how the lack of minute observation, the exercise of a too vivid imagination, and the close reasoning from analogy may lead one astray, while the proper use of these functions may bring out the truth.

Kölliker, Bütschli, Engelmann, and Hertwig, with many others (1870-80) finally demonstrated fully the unicellular nature of the protozoa.

The most important characteristic of a protozoön, its life history, was first partially made out by Trembley in 1744-47. Bütschli helped determine the sexual activities of the members of this group, while Maupas (1889) was the first to demonstrate the conditions leading to their conjugation.

From the earliest investigations into the life history and properties of germs, microorganisms have been thought to play an important part in the causation of infectious diseases. Shortly after the first investigations into this subject the opinion was advanced that puerperal fever, measles, smallpox, typhus, pleurisy, epilepsy, gout, and many other diseases were due to contagion. In fact, so wide-spread became the belief in a causal relation of these minute organisms to disease that it soon amounted to a veritable craze, and all forms and kinds of diseases were said to be produced in this way upon no other foundation than that these organisms had been found in the mouth and intestinal contents of men and animals and in water.

Among those who were specially conspicuous at this time for their advanced views on the germ theory of infectious diseases was Marcus Antonius Plenciz, a physician of Vienna. This acute observer, who published his views in 1762, maintained that not only were all infectious diseases caused by microorganisms, but that the infective material could be nothing else than a living organism. On these grounds he endeavored to explain the variations in the period of incubation of



the different infectious diseases. He also insisted that there were special germs for each infectious disease by which the specific disease was produced. Plenciz believed, moreover, that these organisms were capable of multiplication in the body, and suggested the possibility of their being conveyed from place to place through the air.

These views, it is true, were largely speculative, and rested upon insufficient experiment, but they were so plausible, and the arguments put forward in their support were so logical and convincing, that they continued to gain ground, in spite of considerable opposition and ridicule, and in many instances the conclusions reached have since been proved to be correct. The mode of infection, its unlimited development among large numbers of individuals, and gradual spread over wide areas—the incubation, course of, and resulting immunity in recovery from infectious diseases—all pointed to a living organism as the probable cause.

Among other distinguished men of the day whose observations exerted a most powerful influence upon the doctrine of infection, may be mentioned Henle. His writings (*Pathological Investigations*, 1840, and *Text-book of Rational Pathology*, 1853), in which he described the relation of microorganisms to infectious diseases, and defined the character and action of bacteria upon certain phases and symptoms of these affections, are remarkable for their clearness and precision.

**Origin of Microorganisms.**—But, meanwhile, the question which most interested these investigators into the cause of infectious disease was: Whence are these microorganisms derived which were supposed to produce them? Were they the result of spontaneous generation due to vegetative changes in the substances in which the organisms were found, or were they reproduced from similar preëxisting organisms—the so-called vitalistic theory? This question is intimately connected with the investigations into the origin and nature of fermentation and putrefaction.

Spallanzani, in 1769, demonstrated that if putrescible infusions of organic matter were placed in hermetically sealed flasks and then boiled, the liquids were sterilized; neither were living organisms found in the solutions, nor did the infusions decompose; they remained unchanged for an indefinite period.

The objection was raised to these experiments that the high temperature to which the liquids had been subjected so altered them that spontaneous generation could no longer take place. Spallanzani met the objection by cracking one of the flasks and allowing air to enter, when living organisms and decomposition again appeared in the boiled infusions.

Another objection raised by the believers in spontaneous generation was that, in excluding the oxygen of the air by hermetically sealing the flasks, the essential condition for the development of fermentation which required free admission of this gas was interfered with. This objection was then met by Schulze, in 1836, by causing the air admitted to the boiled decomposable liquids to pass through strong sulphuric

acid. Air thus robbed of its living organisms did not produce decomposition.

Schwann, in 1839, obtained similar results in another way: he deprived of microörganisms the air admitted to his boiled liquids by passing it through a tube which was heated to a temperature high enough to destroy germs. To this investigator is also due the credit of having discovered the specific cause—the yeast plant, or *Saccharomyces cerevisia*—of alcoholic fermentation, the process by which sugar is decomposed into alcohol and carbonic acid.

Again it was objected to these experiments that the heating of the air had perhaps brought about some chemical change which hindered the production of fermentation. Schroeder and von Dusch, in 1854, then showed that by a simple process of filtration, which has since proved of inestimable value in bacteriological work, the air can be mechanically freed from germs. By placing in the mouth of the flask containing the boiled solutions a loose plug of cotton, through which the air could freely circulate, it was found that all suspended micro-organisms could be excluded, and that air passed through such a filter, whether hot or cold, did not cause fermentation of boiled infusions.

Similar results were obtained by Hoffmann, in 1860, and by Chevreul and Pasteur, in 1861, without a cotton filter, by drawing out the neck of the flask to a fine tube and turning it downward, leaving the mouth open. In this case the force of gravity prevents the suspended bacteria from ascending, as there is no current of air to carry them upward through the tube into the flask containing the boiled infusion.

These facts have since been practically confirmed on a large scale in the preservation of food by the process of sterilization. Indeed, there is scarcely any biological problem which has been so satisfactorily solved or in which such uniform results have been obtained; but all through the experiments of the earlier investigators irregularities were constantly appearing. Although in the large majority of cases it was found possible to keep boiled organic liquids sterile in flasks to which the oxygen of the air had free access, the question of spontaneous generation still remained unsettled, inasmuch as occasionally, even under the most careful precautions, decomposition did occur in such boiled liquids.

This fact was explained by Pasteur, in 1860, by experiments showing that the temperature of boiling water was not sufficient to destroy all living organisms, and that, especially in alkaline liquids, a higher temperature was required to insure sterilization. He showed, however, that at a temperature of 110° to 112° C., which he obtained by boiling under a pressure of one and one-half atmospheres, all living organisms were invariably killed.

Pasteur, at a later date (1865), demonstrated the fact that the organisms which resist boiling temperature are, in fact, reproductive bodies, which are now known as *spores*.

In 1876 the development of spores was carefully investigated and explained by Ferdinand Cohn. He, and a little later Koch, showed

that certain rod-shaped organisms possess the power of passing into a resting or spore stage, and when in this stage they are much less susceptible to the injurious action of higher temperatures than in their normal vegetative condition.

When and how life began no one is yet able to say. That spontaneous generation may even be taking place now under unknown conditions is conceivable, but all such ideas are purely hypothetical and there is no evidence that under present conditions any of the known microorganisms have originated in any way except from a previous similar cell.

Stimulated by the establishment of the fact, through Pasteur's investigations, that fermentation and putrefaction were due to the action of living organisms reproduced from similar preëxisting forms, and that each form of fermentation was due to a special microorganism, the study of the causal relation of microorganisms to disease was taken up with renewed vigor. Reference has already been made to the opinions and hypotheses of the earlier observers as to the microbic origin of infectious diseases. The first positive grounds, however, for this doctrine, founded upon actual experiment, were the investigations into the cause of certain infectious diseases in insects and plants. Thus, Bassi, in 1837, demonstrated that a fatal infectious malady of the silkworm—*pébrine*—was due to a parasitic protozoön. Pasteur later devoted several years' study to an exhaustive investigation into the same subject; and in like manner Tulassee and Kühne showed that certain specific affections in grains, in the potato, etc., were due to the invasion of parasites.

Very soon after this it was demonstrated that microorganisms were probably the cause of certain infectious diseases in man and the higher animals. Davaine, a famous French physician, has the honor of having first demonstrated the causal relation of a microorganism to a specific infectious disease in man and animals. The anthrax bacillus was discovered in the blood of animals dying from this disease by Pollender in 1849 and by Davaine in 1850; but it was not until 1863 that the last-named observer demonstrated by inoculation experiments that the bacillus was the cause of anthrax.

The next discoveries were those relating to wounds and the infections to which they are liable. Rindfleisch, in 1866, and Waldeyer and von Recklinghausen, in 1871, were the first to draw attention to the minute organisms occurring in the pyemic processes resulting from infected wounds, and occasionally following typhoid fever. Further investigations were made by Billroth, Fehleisen, and others, in erysipelatous inflammations secondary to injury, who agreed that in these conditions microorganisms could almost always be detected in the lymph channels of the subcutaneous tissues.

The brilliant results obtained by Lister, in 1863-1870, in the antiseptic treatment of wounds to prevent or inhibit the action of infective organisms, exerted a powerful influence on the doctrine of bacterial infection, causing it to be recognized far and wide and gradually lessen-

ing the number of his opponents. Lister's methods were suggested to him by Pasteur's investigations on putrefaction.

In 1877 Weigert and Ehrlich recommended the use of the aniline dyes as staining agents and thus made possible a more exact microscopic examination of microorganisms in cover-glass preparations.

In the year 1880 Pasteur published his discovery of the bacillus of fowl cholera and his investigations upon the attenuation of the virus of anthrax and of fowl cholera, and upon protective inoculation against these diseases. In the meantime he showed that pure cultures might be obtained by the dilution method.

Laveran, in the same year, announced the discovery of parasitic bodies, the first pathogenic protozoa described, in the blood of persons sick with malarial fever, and thus stimulated investigations upon the immensely important unicellular animal parasites.

In 1881 Koch published his fundamental researches upon pathogenic bacteria. He introduced solid culture media (agar had already been used by Frau Hess) and the "plate method" for obtaining pure cultures, and showed how different organisms could be isolated, cultivated independently, and, by inoculation of pure cultures into susceptible animals, could be made, in many cases, to reproduce the specific disease of which they were the cause. To him more than to any other are due the methods which have enabled us to prove absolutely, in a broad sense, the permanence of bacterial varieties. It was in the course of this work that the Abbé system of substage condensing apparatus was first used in bacteriology. Koch's postulates, namely, that (1) a specific organism always associated with a disease (2) when isolated in pure culture (3) and inoculated into a healthy susceptible animal always produces the disease, and (4) may be obtained again in pure culture, were long accepted as the only proof of the causal relationship of that organism to the disease. Later it was learned that immunological reactions added greatly to our knowledge of the specificity of microbes in disease.

In 1882 Pasteur published his first communication upon rabies. The method of treatment devised by him is still in general use. A little later came the investigations of Löffler and Roux upon the diphtheria bacillus and its toxins, and that of Kitasato upon tetanus. These researches paved the way for Behring's work on diphtheria anti-toxin, which in its turn stimulated investigation upon the whole subject of immunity. The number of investigators rapidly increased as the importance of the earlier fundamental discoveries became apparent. Additions to the science of microbiology have been made from many sides, and the practical application of the facts learned from these investigations is steadily increasing. The most important of these are given in the following pages.

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VALLERI, RADOT: Life of Pasteur.

## CHAPTER II.

### CLASSIFICATION AND GENERAL CHARACTERISTICS OF MICROÖRGANISMS.

IN general the lowest forms of life are microscopic. Among these microscopic organisms are many which have in common the ability to produce disease in the higher animals and plants. Some of these disease-producing or pathogenic microörganisms are plants, others are animals, while some are difficult to classify. Only those that are of direct importance to man are considered in this work.

#### CLASSIFICATION OF MICROÖRGANISMS.

The classification of microörganisms is still in the transition stage. This is due chiefly to the difficulties encountered in studying the individual morphological characteristics of such minute bodies and in determining their limits of variation.

There is no one distinctive characteristic known which separates the lowest plants from the lowest animals. While the lowest micro-organisms of all—the bacteria—are usually classed as plants, their structure is so simple and their biological characteristics are so varied that their relationship to the vegetable kingdom is not clear-cut. In their possession of more or less rigid plasmolysible bodies, in the tendency of many to grow in filaments, and in the ability of some to use simple elements as food, they resemble plants; while in the motility of many, the non-possession by all of chlorophyl, and in the necessity of many for complex food, they resemble animals.

There is a similar difficulty in definitely classifying many members of the other groups of closely related microörganisms, namely, the protozoa, the yeasts, and molds, and it has been suggested that under the name *Protista* a third kingdom be formed consisting of all of these lowest microörganisms.

As a rule, in the more minute organisms genera are based upon morphological characteristics, and species upon biochemical, physiological, and pathogenic properties. This is due to the fact that the morphology of varieties may vary extremely under different conditions and that it may give no indication whatever of the relation of micro-organisms to disease and fermentation—the chief characteristic of importance to human beings.

The properties of bacteria, for example, which are fairly constant under uniform conditions and which have been more or less used in systems of classification, are those of spore and capsule formation,

motility (flagella formation), reaction to staining reagents; relation to temperature, to oxygen, and to other food material, and, finally, their relation to fermentation and disease. But any one of these properties, in many groups of microörganisms, such as the coli and the streptococcus groups, under certain conditions may so vary that, taking it as a basis for classification, an organism might be dropped from the group with which it had been classified and be placed in an entirely different group. Thus, the power to produce spores or flagella may be held in abeyance for a time or may be totally lost; the relations to oxygen may be gradually altered, so that an anaërobic bacterium may grow in the presence of oxygen or the contrary may be the case; parasitic organisms may be so cultivated in the test-tube as to become saprophytic varieties, and those which when taken from their natural habitat have no power to grow in the living body may be made to acquire pathogenic properties, by causing the germs to develop in a series of animals of the same species whose resistance has been overcome by reducing their vitality through poisons or other means, or by giving enormous doses of germs at first.

**Permanence of Species.**—It is true, however, that certain microörganisms retain some of their characteristics, so far as we know, for an indefinite time. While we cannot believe that the multitude of varieties which now exist have always existed, and we may theorize as to the part played by the fluctuating variations just mentioned in producing permanent species, the fact observed in the few years during which bacteria have been studied is that most pathogenic species as observed in disease have remained practically unaltered. The diphtheria bacilli are the same today as when Löffler discovered them in 1884, and the disease itself is evidently the same as history shows it to have been before the time of Christ. The same permanence of disease type is true for tuberculosis, smallpox, hydrophobia, leprosy, etc. Under practically unchanged conditions, therefore, such as exist in the bodies of men, most germs which have once become established as parasites continue to reproduce new generations which retain their peculiar (specific) characteristics. It is true that among the countless organisms developed some fail to hold the parasitic characteristics. These either continue as saprophytes or cease to exist. That new pathogenic disease varieties are coming into existence from time to time is, of course, a possibility, but not a certainty. Such *true* mutations have been from time to time reported, notably by Penfold for the coli group, but whether or not the changed characteristics may be considered species characteristics cannot at present be decided.

Our lack of more definite knowledge in regard to significance of these changes, as we have said, is the chief cause of other many unsatisfactory results from attempts at classification.

At present a systematic effort is being made by the Society of American Bacteriologists to formulate rules for the adoption of a classification that will be accepted by all. Until such a universally

accepted classification can be determined it seems wiser to note only such a broad, simple grouping as the one given below.

#### WORKING CLASSIFICATION OF PATHOGENIC MICROÖRGANISMS IN OUTLINE.

Kingdom.	Subkingdom.	Classes in which pathogenic species occur.	Genera in which chief pathogenic species occur.
Plants (fungi)	Molds (Hyphomycetes)	Mycomycetes	Aspergillus, Penicillium.
		Phycomycetes	Mucor.
	Yeasts (Blastomycetes)	Unclassified (Fungi Imperfecta)	Microspora, Trichophyta, Sporotricha, Achoria.
		Oldia	Oidium.
Animals (Protozoa)	Bacteria (Schizomycetes)	Saccharomycetes	Saccharomyces.
		Cocci (Coccaceæ)	Micrococcus, Diplococcus, Streptococcus, Tetracoccus.
	Flagellates (Mastogophora)	Bacilli (Bacteriaceæ)	Bacillus (Bacterium).
		Spirilla (Spirillaceæ)	Spirillum[Spirocheta, Treponema].
	Amebæ (Sarcodina)	Higher bacteria (Trichobacteria)	Cladothrix, Nocardia (Streptothrix).
		Flagellata	Cercomonas, Leptomonas, Herpetomonas, Trypanosoma, Leishmania, Trichomonas, Lamblia [Spirocheta, Treponema].
	Sporozoa	Rhizopoda	Entameba.
		Telosporidia, Neosporidia	Eimeria, Hemogregarinida, Proteosoma, Hemameba, Babesia, Rhinosporidium, Myxobolus, Nosema, Sarcocystis, etc.
	Ciliates	Ciliata	Balantidium.

Unclassified: Ultramicroscopic organisms.

**Nomenclature.**—It is well to call attention to the fact that in naming species, especially among the bacteria, the binomial law of nomenclature has been frequently violated. Such names as *Bacillus coli communis* should not be accepted; the name *Bacillus coli* is sufficient as well as correct.

#### GENERAL CHARACTERISTICS OF EACH GROUP OF MICROÖRGANISMS.

**Introduction.**—The knowledge gained from the fact that each variety of microbe possible of cultivation may grow in distinctive ways upon so-called artificial culture media has been an immense aid in studying

#### EXPLANATION OF PLATE I.

Partly schematic. Rearranged and drawn by Williams from the indicated authors.

FIG. 1.—*Aspergillus glaucus*. Fruiting hyphæ growing from mycelium: A, conidiophore; B, sterigma; C, conidia; D, beginning perithecium; E, conidiophore bearing spores; F, perithecium containing rudiments in section; G, ascus containing eight spores (De Bary).

FIG. 2.—*Penicillium*, showing formation of conidia, A.

FIG. 3.—*Mucor mucedo*: A, sporangium containing spores; B, spores liberated; C, chlamydospores; D, E, F, stages in the formation of a zygospore.

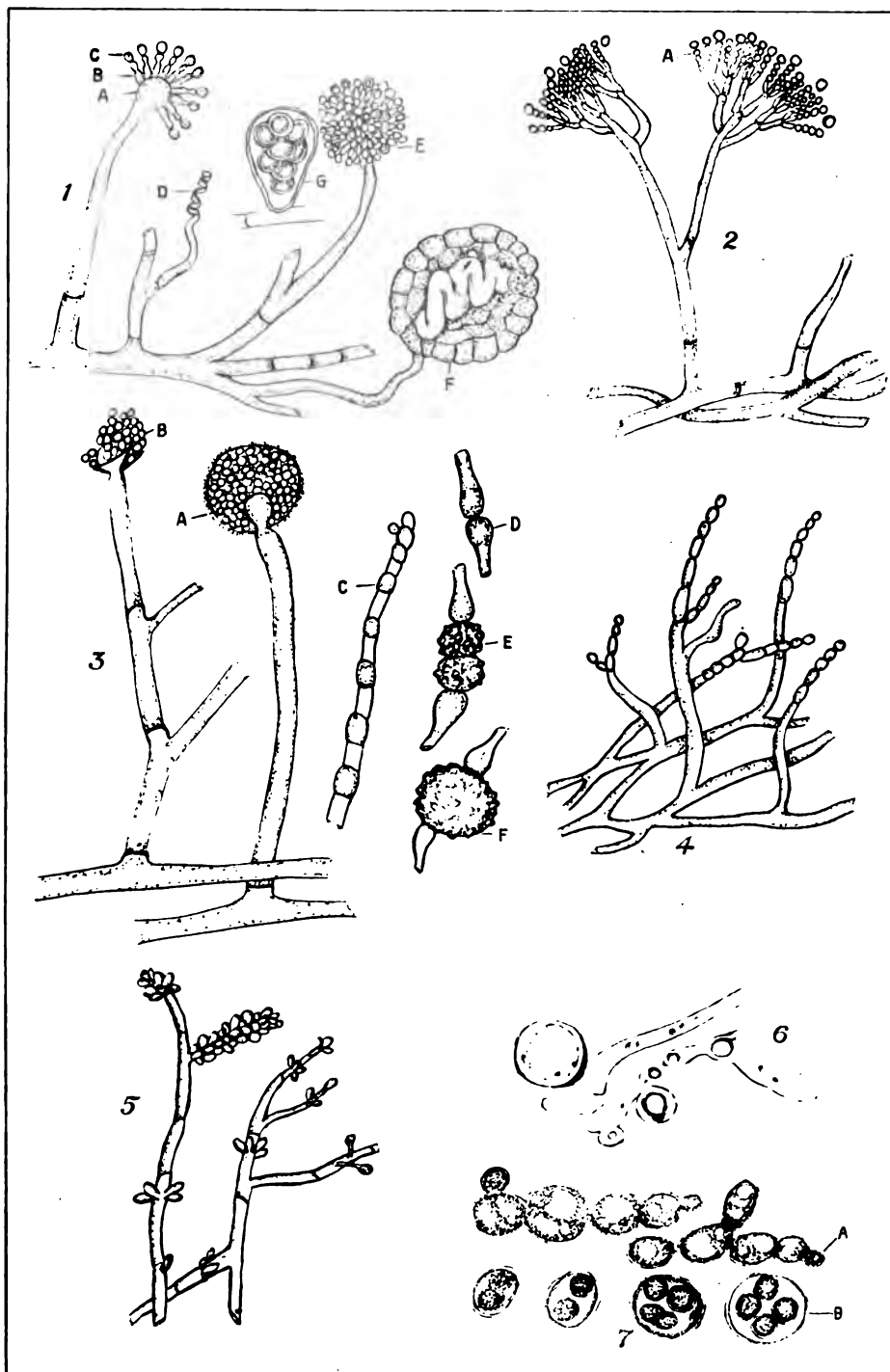
FIG. 4.—*Oldium lactis*.

FIG. 5.—*Sporotrichum schenki*, showing formation of whorled spores on branched mycelium.

FIG. 6.—Yeast from human infection in culture showing mycelium-like growth.

FIG. 7.—*Saccharomyces cerevisiæ* (Hansen): A, budding; B, spore formation.

# PLATE I







the characteristics of the more minute forms, for the individual cell of most varieties is so minute that even the highest magnification we have may show little if any morphological difference between organisms which produce distinctly different diseases, or between a pathogenic and a non-pathogenic form. There are, however, certain morphological and biological characteristics of the single cell which are pronounced, and we therefore study these before going on to the study of cultures, that is, of microorganisms in masses.

Morphological descriptions should always be accompanied by a definite statement of the habitat—if from a culture, age of the growth, the medium from which it was obtained, and the temperature at which it was developed.

### MOLDS (HYPHOMYCETES, EUMYCETES).

The molds (see Plate I) are closely related to the higher bacteria. Like them they grow in branching filaments or threads forming a mycelium, but these filaments or hyphæ, as they are called, are more definite than those of the trichobacteria, and they are multicellular; that is, the filaments are septate, in some varieties always (mycomycetes) and in others when forming spores (phycomycetes). These two groups of molds have other differential characteristics, particularly in their fruiting organs.

The *mycomycetes* are divided into two groups: (1) Ascomycetes, which form a spore sac, containing a definite number of spores, a multiple of two, usually eight (ascospores); (2) basidiomycetes, which form a definite spore-bearing cell called the basidium or conidiophore, from which four (usually) conidia or sterigmata protrude (mushrooms, etc.). The sexual fruiting organs are varied.

Yeasts produce a definite sporangium containing a definite number of spores (two or a multiple of two), therefore they are closely related to the ascomycetes.

The *phycomycetes* are characterized by the formation of *no definite* basidium or ascus. The asexual fruiting organs are formed in three different ways:

1. Ends of hyphæ swell and are shut off by septum, forming *sporangium* in which (a) swarm spores develop. These become free by rupture of sporangium wall, swim about, and then form a new plant; (b) spores form a cell wall while in sporangium.

2. Spores produced directly by constriction at end of certain hyphæ. Such spores are called conidia and the hyphæ conidiophores; each conidium is able to develop into a new plant.

3. Spores produced by separation of hyphæ into short-walled segments or oidia. When the spores are thick-walled they are called chlamydospores.

Sexual reproduction occurs in one of two ways:

1. The ends of two similar hyphæ become attached, then they segment from the rest of the hyphæ and become surrounded by a

thick wall. The partition in the centre dissolves and the protoplasm mixes. The resulting body is called a zygosporé. It produces a new plant. The members of this group are called zygomycetes.

2. The swollen ends of two dissimilar hyphæ, one large (female) and one smaller (male), become attached, fuse, segment, and become surrounded by a thick wall. The body is called an oösporé. The plants are called oömycetes.

*Fungi Imperfecti*.—The molds that are not definitely classified are divided into two groups:

1. Known forms which do not fit in the above group.
2. Not fully known forms. To this group belong most of the molds causing human disease.

### YEASTS (BLASTOMYCETES).

These microörganisms have been for many centuries of the greatest importance in brewing and baking. They are not uncommonly present in the air and in cultures made from the throat. Certain recent experiments have shown that some varieties when injected are capable of producing tumor-like growths. Certain varieties are pathogenic for mice, and in recent years (since 1894) there have been reported a number of cases of human infection from yeasts.

The position which the yeasts occupy in systematic biology has not, thus far, been accurately determined. In fact, it is still a question as to whether they constitute distinct fungi or whether they should be classed under the molds.

The chief morphological characteristic of the yeasts is their frequent and marked method of reproduction by means of budding. For this reason these organisms go by the name of *blastomycetes* in contrast to the fission fungi, or *schizomycetes*, and the thread fungi, or *hyphomycetes*. The fact was mentioned above that some yeasts form ascospores and that they are therefore related to the ascomycetes. The *soor fungus*, which at one time grows in long threads, at another time (under certain conditions almost exclusively) multiplies by budding, is also a transition form. It is thus seen that no hard-and-fast line exists between yeasts and molds.

The most important property of yeasts, though one not possessed by all to the same degree, is that of producing alcoholic fermentation. In brewing we distinguish between the yeasts that can be employed practically, "culture yeasts," and those which often act as disturbing factors, so-called "wild" yeasts. The enzyme causing this action is called zymase.

The shape of most of the culture yeasts is oval or elliptic (Plate I, Fig. 7). Round or globular forms are more often met with among the wild species which usually excite only a slight degree of fermentation. They are known as "torula" forms. But sausage-shaped and thread forms are also met with. Pathogenic forms may also be round.

The individual yeast cells are strongly refractive, so that under the microscope at times they have almost the lustre of fat droplets. This is important because in examining fresh tissues the yeast cells may be hard to distinguish from fat droplets, often requiring the aid of certain reagents for their identification.

The size of the individual yeast cells varies enormously, even in those of the same species or the same culture. In old colonies individuals may be found no larger than cocci,  $0.5\mu$  to  $1\mu$  in diameter, while in other colonies, especially on the surface of a liquefied medium, giant yeast cells are found often attaining a diameter of  $40\mu$  or more. In spite of these wide fluctuations, however, the various species are characterized by a fairly definite average in size and form. Each cell contains a more or less definite nucleus, which is demonstrated by the usual chromogenic stains.

During the process of budding the nucleus of the cell moves toward the margin, where it divides. At this point the limiting membrane of the cell ruptures or usually a hernia-like protrusion develops which has the appearance of a button attached to the cell. The daughter-cell so formed rapidly increases in size and gradually assumes the shape and size of the mother-cell.

A fact of the utmost importance for the propagation of the blastomycetes and continuation of the species is the formation of spores (Plate I, Fig. 7). In this also the cell nucleus takes part, dividing into several fragments, each of which becomes the centre of a new cell lying within the original cell. These new cells possess a firm membrane, a cell nucleus, and a little dense protoplasm. The number of spores developed in the yeast cells varies, but is constant for a given species. As a rule, one cell does not produce more than four *endogenous* spores, but species have been observed, *e. g.*, *Schizosaccharomycès octosporus* (Beijerinck), in which eight spores are found.

Guilliermond has described conjugation in yeasts before the formation of spores.

The vitality of yeasts is truly enormous. Hansen as well as Lindner were able to obtain a growth from cultures twelve years old. Busse succeeded in getting a luxuriant growth from a dry potato culture seven and a half years old, which was almost as hard as bone.

## BACTERIA.

**Definition.**—*Bacteria* may be defined as extremely minute, simple, unicellular microorganisms, which reproduce themselves with exceeding rapidity, usually by transverse division, and grow without the aid of chlorophyl. They have no morphological nucleus, but contain nuclear material which is generally diffused throughout the cell body in the form of larger or smaller granules.

**Natural Habitat.**—There are such wonderful differences in the conditions of life and nutrition which suit the different varieties, that bacteria are found all over the known world. Wherever there is sufficient

moisture, one form or another will find other conditions adequate for multiplication. Thus, we meet with bacterial life between 0° and 75° C. Some live only in the tissues of men, others in lower animals, a large number may grow in both man and lower animals, others still grow only in plants, but by far the greater number live in dead organic matter. For some free oxygen is necessary to life; for others it is a poison.

**Morphology** (see Plates II and III).—The form and dimensions of bacterial cells at their stage of complete development must be distinguished from those which they possess just after or just before they have divided. As a spherical cell develops preparatory to its division into two cells it becomes elongated and appears as a short oval rod; at the moment of its division, on the contrary, the transverse diameter of each of its two halves is greater than their long diameter. A short rod becomes in the same way, at the moment of its division, two cells, the long diameter of each of which may be even a trifle less than its short diameter, and thus they appear on superficial examination as spheres.

**Size.**—The dimensions of the adult individual vary greatly in the different species as well as in members of the same species. The largest bacillus recorded is  $50\mu^1$  to  $60\mu$  long and  $4\mu$  to  $5\mu$  wide (*B. bütschlii*, see Fig. 7). One of the smallest forms known (*B. influenzae*) has an average size of  $0.5\mu \times 0.2\mu$ .

Some pathogenic organisms are so small (ultramicroscopic, see p. 70, also chapter on Filtrable Viruses) as to be invisible with any magnification which we now possess. We know of their existence either by the fact that they may be cultivated on artificial media, producing appearances of mass growth, and that such cultures when inoculated into susceptible animals cause the characteristic disease, or by the fact

#### EXPLANATION OF PLATE II.

FIG. 1.—Illustrating cocci single or in irregular groups (micrococcus, staphylococcus), micrococcus from air.  $\times 1000$ .

FIG. 2.—Illustrating cocci in twos—*Diplococcus pneumoniae* from peritoneal exudate of rabbit.  $\times 1000$ .

FIG. 3.—Illustrating cocci in chains—*Streptococcus pyogenes*.  $\times 1000$ .

FIG. 4.—Illustrating cocci in fours—*Micrococcus tetragenus* from spleen of mouse.  $\times 1000$ .

FIG. 5.—Illustrating cocci in packets—*Sarcina lutea* from air.  $\times 1000$ .

FIG. 6.—Illustrating large single bacilli: *B. subtilis* (hay bacillus).  $\times 1000$ .

FIG. 7.—Illustrating small bacilli; mostly in twos—*B. hoffmanni* from human throat  $\times 800$ . (Park.)

FIG. 8.—Illustrating bacilli in chains—*Anthrax bacillus* from spleen of mouse.  $\times 500$ .

FIG. 9.—Illustrating bacilli in bunches—*Typhoid bacillus* from human spleen.  $\times 500$ .

FIG. 10.—Illustrating bacilli in threads—*Anthrax bacilli* from blood of frog.

FIG. 11.—*Spirillum undula*, showing flagella.  $\times 1000$ .

FIG. 12.—*Cholera spirilla*, gelatin culture.  $\times 1000$ .

FIG. 13.—Large spirilla in chains from water.  $\times 1000$ .

FIG. 14.—Smaller spirilla in chains—*Spirillum rubrum*.  $\times 1000$ .

FIG. 15.—*Streptothrix candida*—Broth culture (Zettnow, from Kolle and Wassermann).

FIG. 16.—*Streptothrix hominis* from sputum (Zettnow, from Kolle and Wassermann).

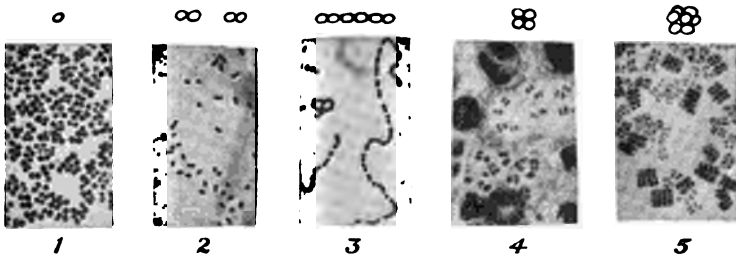
Unless otherwise indicated, the photographs are from Fränkel and Pfeiffer.

<sup>1</sup> A  $\mu$ , or micromillimeter, is  $\frac{1}{25000}$  of an inch.

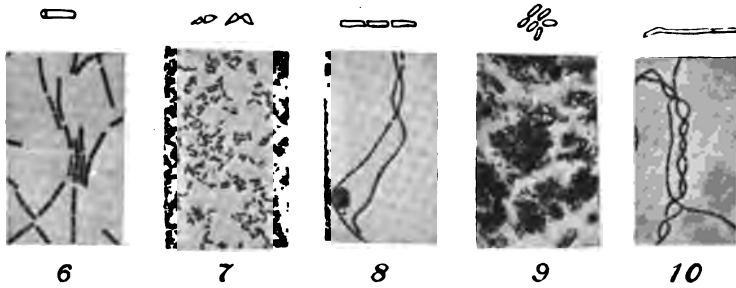
# PLATE II

## TYPES OF BACTERIA

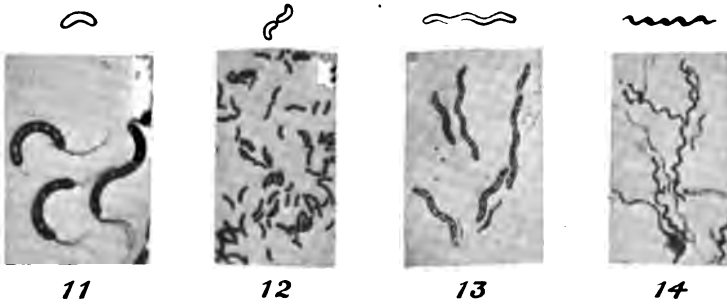
### I SPHERE (COCCI OR COCCACEÆ)



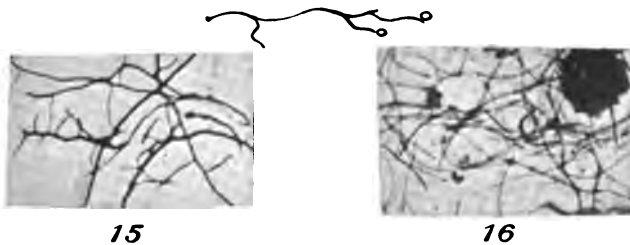
### II CYLINDER (BACILLI OR BACTERIACEÆ)



### III SPIRAL (SPIRILLA OR SPIRILLACEÆ)



### IV HIGHER BACTERIA (TRICHOBACTERIA)





that the filtrates alone are infectious. Some filtrates are infectious after passing through the pores of the finest filter.

**Shape.**—The *basic forms* of the single bacterial cells are threefold—the sphere, the rod, and the segment of a spiral. Although under different conditions the type form of any one species may vary considerably, yet these three main divisions under similar conditions are constant, and, so far as we know, it is never possible, by any means, to bring about changes in the organisms that will result in the permanent conversion of the morphology of the members of one group into that of another—that is, micrococci always, under suitable conditions, produce micrococci, bacilli produce bacilli, and spirilla produce spirilla.

As bacteria multiply, the cells produced from the parent cell have a greater or less tendency to remain attached. This is on account of the slimy envelope which is more or less developed in all bacteria. In some varieties this tendency is extremely slight, in others it is marked. This union may appear simply as an aggregation of separate bacteria or so close that the group appears as a single cell. According to the method of the cell division and the tenacity with which the cells hold together, there are different groupings of bacteria, which aid us in their differentiation and identification. Thus, in cocci we get the bacterial cell dividing into one, two, or three planes (Plate II, Figs. 1-5), while in bacilli and spirilla the division is generally in only one plane (Plate II, Figs. 6-9).

1. **SPHERICAL FORM, OR COCCUS.**—The size varies from about  $0.15\mu$  as minimum diameter to  $3\mu$  as maximum; the average size of the pathogenic cocci is  $0.8\mu$ . The single elements are, at the moment of their complete development, so far as we can determine, practically spherical; but when seen in the process of multiplication through division the form is seldom that of a true sphere. Here we have elongated or lancet-shaped forms, as frequently seen in the diplococcus of pneumonia, or the opposite, as in the diplococcus of gonorrhea, where the cocci appear to be flattened against one another. Those cells which divide in one direction only and remain attached are found in pairs (diplococci) or in shorter or longer chains (streptococci). Those which divide in two directions, one at right angles to the other, form groups of four (tetrads). Those which divide in three directions and cling together form packets in cubes (sarcinae). Those which divide in any axis form irregularly shaped, grape-like bunches (staphylococci).

2. **ROD FORM, OR BACILLUS.**—The type of this group is the cylinder. The length of the fully developed cell is always greater than its breadth. The size of the cells of different varieties varies enormously: from a length of  $30\mu$  and a breadth of  $4\mu$  to a length of  $0.2\mu$  and a breadth of  $0.1\mu$ . The largest bacilli met with in disease do not, however, usually develop over  $3\mu \times 1\mu$ , while the average is  $2\mu \times 0.5\mu$ . Bacilli are roughly classed, according to their form, as slender when the ratio of the long to the transverse diameter is from 1 to 4 to 1 to 10, and as thick when the proportions of the long to the short diameter is approximately 1 to 2.

The characteristic form of the bacillus has a straight axis, with



uniform thickness throughout, and flat ends; but there are many exceptions to this typical form. Thus, frequently the motile bacteria have rounded ends, many of the more slender forms have the long axis, slightly bent; some few species, as for example the diphtheria bacilli, invariably produce many cells whose thickness is very unequal at different portions. Spore formation also causes an irregularity of the cell outline.

The bacilli, except when they develop from spores or granules, divide only in the plane perpendicular to their long axis. A classification, therefore, of bacilli according to their manner of grouping is much simpler than in the case of the cocci. We may thus have bacilli as isolated cells, as pairs (diplobacilli), or as longer or shorter chains (streptobacilli).

3. SPIRAL FORM, OR SPIRILLUM.—The members of the third morphological group are spiral in shape, or only segments of a spiral. Here, too, we have large and small, slender and thick spirals. The twisting of the long axis, which here lies in two planes, is the chief characteristic of this group of bacteria. Under normal conditions the twisting is uniform throughout the entire length of the cell. The spirilla, like the bacilli, divide only in one direction. A single cell, a pair, or the union of two or more elements may thus present the appearance of a short segment of a spiral or a comma-shaped form, an S-shaped form, or a complete spiral or corkscrew-like form.

#### EXPLANATION OF PLATE III.

Partly schematic (Williams). Stained by Giemsa except where otherwise indicated.

Figs. 1 to 4.—Anthrax bacilli at different stages of development.

FIG. 1.—Shows chromatin-staining masses (red), in plastin-staining cells (light blue).

FIG. 2.—Shows same organism after one hour on fresh medium, chromatic substance distributed throughout medium and whole organism taking deeper, more homogeneous stain.

FIG. 3.—Organism showing two kinds of granules according to staining powers.

FIG. 4.—Same organism placed on fresh medium extrudes some granules (waste-products) and redissolves others (chromatin granules).

Figs. 5 to 9.—Similar granules in diphtheria bacilli at various stages of development.

FIG. 10.—Metachromatic granules in *B. pyocyaneus*, Neisser stain.

FIG. 11.—Metachromatic granules in *Sarcinae*, Neisser stain.

FIG. 12.—Metachromatic granules in *B. influenzae*, Giemsa's stain.

FIG. 13.—Metachromatic granules in gonococci, Giemsa's stain.

FIG. 14.—Pneumococcus, capsule stained by Hiss method.

FIG. 15.—Pneumococcus mucosus (*Streptococcus mucosus*) by Welch stain for capsule.

FIG. 16.—Rhinoscleroma bacillus by Hiss stain for capsules.

FIG. 17.—Plasmolysis in cholera bacillus showing capsule.

Figs. 18 to 21 inclusive.—Types of flagella by Löffler stain.

FIG. 18.—Monotricha, cholera spirillum.

FIG. 19.—Amphitricha, water bacillus.

FIG. 20.—Lophotricha, spirillum undula.

FIG. 21.—Peritricha, typhoid bacillus.

FIG. 22.—Formation and end germination of spores in anthrax.

FIG. 23.—Lateral germination of spore in *B. subtilis*.

FIG. 24.—Central germination of spores in *B. alvei* (Wilson).

FIG. 25.—Type of spores: *A*, central; *B*, eccentric; *C*, end.

FIG. 26.—Diphtheria bacilli in old cultures, Giemsa's stain.

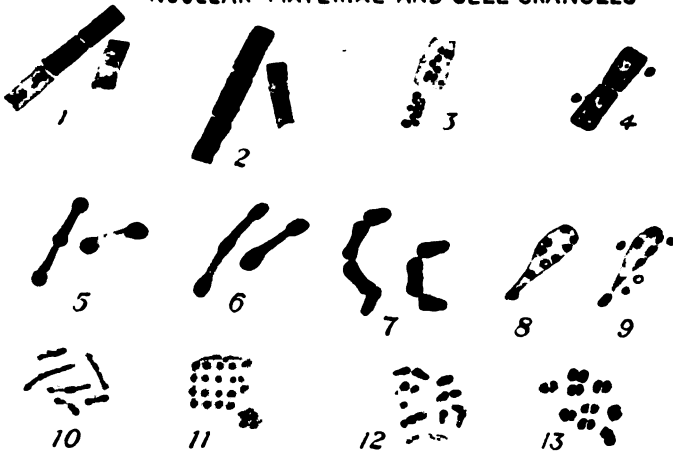
FIG. 27.—Plague bacilli in old cultures, stained by methylene blue.

FIG. 28.—Influenza bacilli in old cultures, Giemsa's stain.

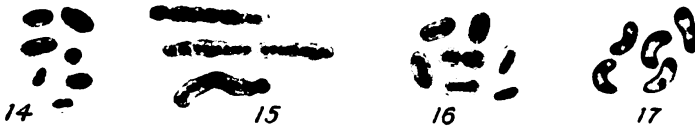
# PLATE III

## STRUCTURE OF BACTERIA

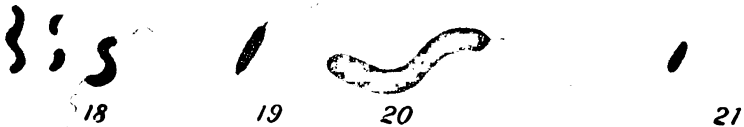
### NUCLEAR MATERIAL AND CELL GRANULES



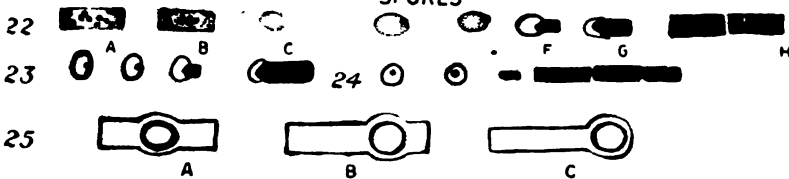
### CAPSULES AND MEMBRANES



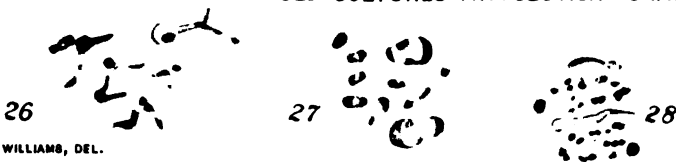
### FLAGELLA



### SPORES



### IRREGULAR FORMS IN OLD CULTURES (INVOLUTION FORMS)





**Structure of Bacterial Cells.**—When examined living in a hanging drop (see p. 71) under the microscope, bacteria appear usually as colorless refractive bodies with or without spores or other more highly refractive areas. It is only by the use of stains that we are able to see more of their structure.

**Capsule.**—Special staining methods (see p. 78) show that many bacteria (some investigators say all) under certain conditions possess a *capsule* (Plate III, Figs. 14–16), a gelatinous envelope which is supposed to be formed from the outer layer of the cell membrane. Some bacteria easily develop a much thicker capsule than others. Such forms are known as capsule bacteria. These generally produce a slimy growth on cultivation (*e. g.*, *B. mucosus*).

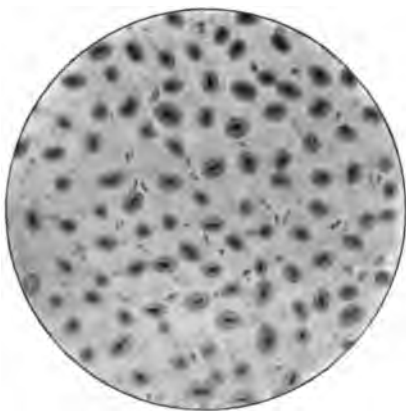


FIG. 1.—*Bacillus pneumoniae* (*B. mucosus*) stained for capsule by Huntoon's method.  $\times$  about 900. (Huntoon.)

Capsules develop best in animal tissues. In cultures, with a few exceptions, they require for their development special albuminous culture media, such as milk, blood serum, bronchial mucus, etc. In ordinary nutrient media or on potatoes the capsule may be visible in the first culture generations when grown from the body, but usually it shows very indistinctly, if at all. The capsule is distinguished by a diminished power of staining with ordinary aniline dyes; therefore, unless special staining methods are used, the bacteria may appear to be lying in a clear unstained area. With certain dyes the inner portion of the capsule stains, giving the bacteria an apparent greater diameter. The demonstration of the capsule is often of help in differentiating between different but closely related bacteria; *e. g.*, some forms of streptococcus and pneumococcus.

**Cell Membrane.**—That all bacteria possess a cell membrane is shown (1) by special staining methods (*e. g.*, flagella stains, see p. 80) and (2) by plasmolysis, demonstrated by placing the bacteria in a 1 per cent. solution of sodium chloride when the central portion (entoplasm?) contracts and separates in places from the membrane (see also p. 57,

under Influence of Pressure). In some bacteria the membrane is slightly developed, while in others (*e. g.*, *B. tuberculosis*) it is well developed. It is different in composition from the membrane of higher plants in not possessing cellulose. In some forms, however, a similar carbohydrate, hemicellulose, has been demonstrated. In certain forms a substance related to chitin, found in the cyst walls of protozoa, has been found. Some observers consider the cell membrane merely a concentrated part of the cytoplasm, similar to the ectoplasm of higher cells. That it is closely related to the living part of the cell is shown by the connection of the organs of locomotion (flagella) with it (Plate III, Figs. 17 and 21).

**The Cell Substance.**—The chief views in regard to the nature and the structure of the cell substance contained within the membrane may be summarized as follows:

1. Bacteria have a definite morphological, more or less centrally situated nucleus (Feinberg, Nakanischi, Shottelius, Swellengrebel, and others).
2. Bacteria have no nucleus or differentiated nuclear material (Fischer, Migula, Massart, and others).
3. The whole organism, except the membrane which is a delicate layer of cytoplasm, is a nucleus (Bütschli, Löwit, Boni, and others).
4. The nuclear material is in the form of distributed chromatin granules throughout the cytoplasm (Hertwig, Schaudinn, Guilliermond, Zettnow, and others).
5. A variety of the fourth view is that bacteria possess both chief elements of a cell, namely, cytoplasm and karyoplasm, but that these are so finely mixed that they cannot be morphologically differentiated (Weigert, Mitrophanow, Gotschlich).
6. The latest view advanced, which is a variation of the views 3, 4, and 5, is that the bacterial cell is a relatively simple body—a cytode in Haeckel's sense, or the plasson of Van Beneden—which possesses both chromatin and plastin, the relative amounts of these chief substances of a cell corresponding more to the amounts found in the nuclei of higher cells than in their cytoplasm (Ruzicka, Ambrôz).

The last two authors call attention to the fact that both nucleus and cytoplasm in the higher cells are composed of a mixture of chromatin and plastin and that the chief difference between the two mixtures is one of amount and not of kind.

Our own studies of the structure of bacteria lead us to agree with the views expressed in Nos. 4 and 6 of the above summary—that is, bacteria possess both chief elements of a cell, namely, chromatin and plastin, and according to the stage of growth and division (varying with species) the chromatin may be in the form of morphological granules or may be so finely divided and mixed with the plastin as to be indistinguishable from it. (See Plate III, Figs. 1, 2, 5, 6, 7.)

**Metachromatic Granules** (Plate III, Figs. 1-13).—These granules appear in unstained bacteria as light-refracting, in stained preparations as deeply stained areas. They have a great affinity for dyes, and so stain readily and give up the stain with some difficulty. With complex stains they show a greater affinity than the rest of the bacillus for certain constituents of the stain—*e. g.*, with polychromic methylene blue they take up more of the azure, thus appearing red as does the stained nuclein in morphological nuclei. In certain bacteria, such as

the diphtheria bacilli, they are especially well marked in young, vigorous cultures. Here they have diagnostic value. At least some of these granules are nuclear in character.

Certain other granules which take up stains readily, and others still which absorb stains with difficulty, are of the nature of starch or fat or of other food products. Meyer has described some as being composed of volutin, a protein characterized by insolubility in alcohol and solubility in water, acids and alkalis. Certain saprophytic forms have sulphur, others iron granules.

**Organs of Motility.**—The outer surface of spherical bacteria is almost always smooth and devoid of appendages; but that of the rods and spirals is frequently provided with fine, hair-like appendages, or *flagella*, which are organs of motility (Plate III, Figs. 18–21). These flagella, either singly or in tufts, are sometimes distributed over the entire body of the cell, or they may appear only at one or both ends of the rod. The polar flagella appear on the bacteria shortly before division. The

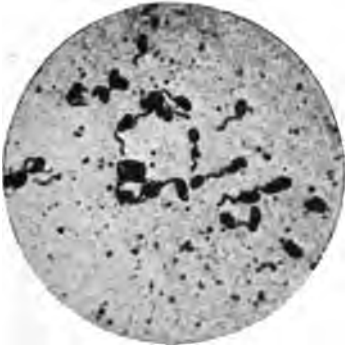


FIG. 2.—Bacilli showing one polar flagellum.



FIG. 3.—Bacilli showing multiple flagella.

flagella are believed to be formed from the outer cell layer (ectoplasm) or possibly from the capsule, though they have been described by certain authors as arising in endoplasmic granules. So far as we know, the flagella are the only means of locomotion possessed by the bacteria. They are not readily stained, as special staining agents are required for this purpose (see p. 80). The envelope of the bacteria, which usually remains unstained with the ordinary dyes, then becomes colored and more distinctly visible than is commonly the case. Occasionally, however, some portion of the envelope remains unstained, when the flagella present the appearance of being detached from the body of the bacteria by a narrow zone. In stained cultures of richly flagellated bacteria peculiar plaited masses sometimes are observed, consisting of flagella which have been detached and then matted together. Bacteria may lose their power of producing flagella for a series of generations. Whether this power be permanently lost or not we do not know.

Bacteria are named according to the number and position of the flagella they possess as follows: *Monotricha* (a single flagellum at one pole, *e. g.*, cholera spirillum); *amphitricha* (a flagellum at each pole, *e. g.*, many spirilla); *lophotricha* (a tuft of flagella at one pole,<sup>1</sup> *e. g.*, *Spirillum undula*); *peritricha* (flagella projecting from all parts of surface, *e. g.*, *B. alvei*, *B. typhosus*, and others) (Plate III, Figs. 18–21).

So far, in only a few bacteria (the largest spirilla) have flagella been demonstrated during life, and then only under special conditions (see K. Reichert for bibliography). We have, however, an organism belonging to the *B. alvei* group, which shows its flagella very distinctly during life when a small portion of the viscid growth in a liquefying Löffler blood-serum tube is transferred to a hanging mass of agar (p. 72) and examined under high magnification. The flagella on this organism may also be seen with dark-field illumination. Reichert claims that all motile bacteria show their flagella by this method.

**Spores.**—These important structures of the bacterial cell are described in detail under Physiological Characteristics of Bacteria.

**Physiological Characteristics of Bacteria.**—The essential physiological activities of bacteria are: motility, growth, reproduction, and spore formation.

**Motility.**—Many bacteria when examined under the microscope are seen to exhibit active movements in fluids. The movements are of a varying character, being described as rotary, undulatory, sinuous, etc. At one time they may be slow and sluggish, at another so rapid that any detailed observation is impossible. Some bacteria are very active in their movements, different individuals progressing rapidly in different directions, while with many it is difficult to say positively whether there is any actual motility or whether the organism shows only molecular movements—so-called “*Brownian*” movements, or *pedesis*—a dancing, trembling motion possessed by all finely divided organic particles. In order to decide definitely with regard to the motility of any bacterial preparation, it is well to make two hanging drops. To one, 5 per cent. of formalin is added, which, of course, kills the organism. If, now, the live culture shows motility, which is not shown by the killed culture, it is an indication that one is dealing with a motile culture. Very young cultures, of but three or four hours’ development, in neutral nutrient bouillon should be examined at a temperature suitable for their best growth. Not all species of bacteria which have flagella exhibit at all times spontaneous movements; such movements may be absent in certain culture media and at too low or too high temperatures, or with an insufficient or excessive supply of oxygen; hence one should examine cultures under various conditions before deciding as to the non-motility of any organism.

The highest speed of which an organism is capable has been approximately estimated with some forms, and the actual figures show an actual slow rate of movement, though, comparatively, when the size of the organism is considered, the movement is rapid. Thus, the

<sup>1</sup> Some investigators consider that every flagellum is essentially a tuft, composed of many small fibrils.

cholera spirillum may travel for a short time at the rate of 18 cm. per hour.

Movement is influenced by many factors, such as chemicals (the oxygen in the air especially), heat, light, and electricity. The tactile property which enables microorganisms to take cognizance of various forces is known as *taxis*; when forces attract, the phenomenon is known as positive taxis and when they repel, it is called negative taxis. Chemotaxis, or the effect of chemicals, is taken up in detail on page 59.

**Growth and Reproduction.**—Under favorable conditions bacteria grow rapidly to a certain size, more or less constant for each species, and then divide by fission into approximately equal halves. The average time required for this cycle is twenty to thirty minutes. Probably in all species the nuclear material divides first. This is certainly the case in the group to which the *B. diphtheriæ* belongs, where division of the nuclear granules may be observed in the living organism before the characteristic snapping of the cell body and where division into equal halves seldom occurs.

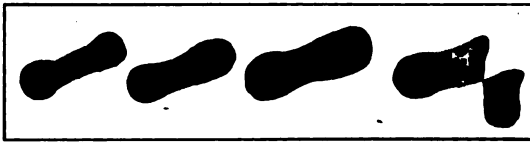


FIG. 4.—Successive stages in division of *B. diphtheriæ*, showing relation of line of division to metachromatic granule. Continuous observation of living bacillus drawn without camera lucida. (Williams.)

According to our observations on the living cell of members of this group, division takes place at a point occupied by a metachromatic granule (Fig. 4). Before division of the cell body the metachromatic granule, which appears to contain nuclear substance, elongates and shows a darker line at or near its centre. This seems to divide and form two lines, each of which has at a point near the surface a very tiny, refractive granule, staining deeply with chromatin stains. Between these two lines the cell body suddenly divides with a snap, like the opening of a jack-knife, division beginning at the point between the two tiny granules, and the two new cells remain for a variable time attached at opposite points, thus giving the V-shaped forms. Kurth and Hill also called attention to division by snapping in members of the diphtheria-bacillus group, though neither recognized the relation between the position of the metachromatic granules and the point of division. The tiny granules are probably similar to the cell-partition granules described by various observers.

It is not often that the favorable conditions mentioned above for the production of equal and rapid division obtain for any time, since even in pure cultures bacteria in their growth soon produce an environment unfavorable for further multiplication. Several factors help to make this environment: (1) The using up of suitable food and moisture; (2) the disintegration of food substances into various injurious products, such as acids, alkalis, ferments; (3) in mixed cultures the overgrowth of one or more varieties. As these unfavorable con-



ditions are more or less constantly present, we seldom see such absolute symmetry in the growth and division of bacteria as is usually described. In fact, except under ideally favorable conditions (*e. g.*, rapid successive transfers from young cultures on the most favorable food medium), we can never see absolutely equal fission among bacteria; and in some species, notably the diphtheria group, division is extremely irregular even in our usual twenty-four cultures on favorable media.

*Involution and Degeneration Forms.*—It follows, from the conditions considered above, that, as cultures grow older or when media unfavorable to equal division are used, the bacteria may show extremely irregular forms, absolutely different from the young forms, such as long threads or filaments with irregular thickenings, coccus forms from bacilli and spirilla which have divided without increasing in length, bacillar forms from cocci which have grown without dividing, and apparently branched forms from many varieties of bacilli and spirilla. These have been called *involution* or *degeneration forms*.

In our study of the so-called branched forms of the diphtheria bacillus we have observed the following interesting fact: Under certain conditions, marked apparent branching appears at a definite time in the age of the culture. The conditions are: slightly disturbed growth in pellicle on nutrient broth. When such pellicles are examined every day they are found to contain, on the sixth to the twelfth day, varying chiefly with the amount of disturbance, many large intensely staining forms with one to several apparent branches and many large metachromatic granules (Figs. 5 and 6). The facts that these forms were the only ones to show active growth and division when examined on a hanging mass of agar and that in such growth the metachromatic granules seem to fuse (Fig. 6) before fission led us to suppose that these forms represent a primitive sexual process, a sort of autogamy. Schaudinn (Fig. 7) has reported a primitive conjugation (autogamy) and a relationship between the chromatin granules, or nuclear substance, and the spores in certain bacteria.

Although elongation in the greater diameter and complete division at right angles to this is the rule for the majority of bacteria, there are certain groups, which, instead of becoming separated from each other as single cells, tend to produce an incomplete segmentation, the cells remaining together in masses, as the sarcinæ, for example, which divide more or less regularly in three directions. The indentations upon these masses or cubes, which indicate the point of incomplete fission, give to these bundles of cells the appearance commonly ascribed to them—that of a bale of rags. The rod-shaped bacteria never divide longitudinally.

*Spore formation* must be distinguished from vegetative reproduction. This is the process by which the organisms are enabled to enter a stage in which they resist deleterious influences to a much higher degree than is possible for them to do in the growing or vegetative condition. It is true that in all non-spore-bearing cultures a certain proportion of the bacteria are more resistant than the average. No marked difference in protoplasm, however, has been noted in them other than the ability to stain more intensely and sometimes to show strong metachromatic areas. The difference between these and the less resistant

forms is not great. Some have believed that this resistance is due to certain bodies called *arthrospores*, which are abnormally large cells with, usually, a thickened cell wall and increased staining properties, formed as a rule in old cultures. Foulerton and others have described similar forms in some of the higher bacteria and consider them spores. (See under *Nocardia*.)



FIG. 5.—*B. diphtheriæ* "No. 8" from 9 days' broth pellicle, showing many "branched" forms. Stained with carbolfuchsin.  $\times 1500$  diameters.



FIG. 6.—*B. diphtheriæ* "No. 8" from 10 days' broth pellicle, showing longitudinal fusion and position of metachromatic granules. Stained with Löffler's methylene blue.  $\times 2000$  diameters.

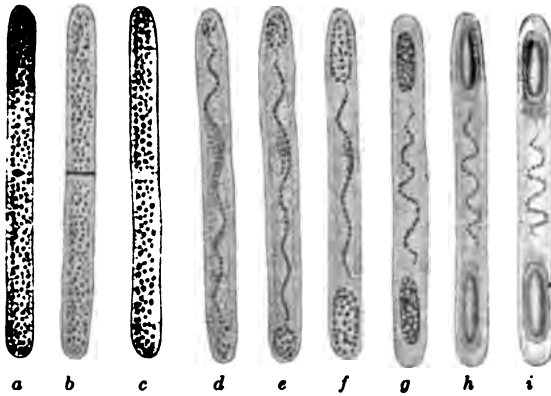


FIG. 7.—*Bacillus butschlii*: *a* to *c*, incomplete division of the cell; *d* to *f*, gradual collection of chromatin granules at ends of cells; *g* to *i*, formation of end spores from these chromatin end masses. (After Schaudinn.)

The *true spores* (endospores) of the lower bacteria are definite bodies. These are strongly refractile and glistening in appearance, oval or round in shape, and composed of concentrated protoplasm developed within the cell and surrounded by a very dense envelope (Plate III, Figs. 22-25). They are characterized by their power of resisting the injurious influences of heat, desiccation, and chemical disinfectants up to a certain limit (see chapter on Disinfection). Spores also stain with great difficulty. (See page 79 for details.)

The production of endospores in the different species of bacteria, though not identical in every instance, is very similar. The conditions under which they are produced in nature are supposed to be similar to those observed in artificial cultures, but they may not always be similar, hence we must not consider a bacterium a non-spore bearer because in the laboratory it has not been seen to form spores. Usually the formation of spores in any species is best observed in a streak culture on nutrient agar or potato, which should be kept at the temperature nearest the optimum for the growth of the organism to be examined. At the end of twelve, eighteen, twenty-four, thirty, thirty-six hours, etc., specimens of the culture are observed, first unstained in a hanging drop or on an agar mass, and then, if round or oval, highly refractile bodies are seen, stained for spores. Each bacillus, as a rule, produces but one spore, and more than two have never been observed.

Motile bacteria usually come to a state of rest or immobility previous to spore formation. Several species first become elongated. The anthrax bacillus does this, and a description of the method of its production of spores may serve as an illustration of the process in other bacteria (Plate III, Fig. 22, A-E). In the beginning, the protoplasm of the elongated filaments is homogeneous, but after a time it becomes turbid and finely granular. These fine granules are then replaced by a smaller number of coarser granules, the so-called sporogenous granules supposed to be chiefly nuclear in nature, which by coalescence finally amalgamate into a spherical or oval refractive body. This is the spore. As soon as the process is completed there may appear between each two spores a delicate partition wall. For a time the spores are retained in a linear position by the cell membranes of the bacilli, but these are later dissolved or broken up and the spores are set free. Not all the cells that make the effort to form spores, as shown by the spherical bodies contained in them, bring these to maturity; indeed, many varieties, under certain cultural conditions, lose altogether their property of forming spores.

The following are the most important spore types: (a) the spore lying in the centre of the cell; which may be much distended in its central portion, giving it a spindle shape or clostridium, *e. g.*, *Bacillus butyricus*; (b) the spore lying at the extremity of a cell much enlarged at that end—the so-called “head spore” or plectridium, *e. g.*, the tetanus bacillus; (c) the spore lying eccentrically (Plate III, Fig. 25, A-C).

According to Schaudinn and others, in certain spore-bearing bacteria the spore formation is part of a sexual-like process. (See under Reproduction.)

The *germination of spores* takes place as follows (Plate III, Figs. 22-24): By the absorption of water they become swollen and pale in color, losing their shining, refractive appearance. Later, a little protuberance is seen upon one side (equatorial or central germination) or at one extremity of the spore (polar germination), and this grows

out to form a rod which consists of soft-growing protoplasm enveloped in a membrane, which is formed of the endosporium or inner layer of the cellular envelope of the spore. The outer envelope, or exosporium, is either cast off when it may be seen in the vicinity of the newly formed rod; or it may be absorbed, as is often the case after central germination.

The chief spore formers among the pathogenic bacteria are the anaërobes (tetanus, malignant edema, intestinal bacteria). Only one distinctly pathogenic aërobe produces endospores—the anthrax bacillus.

**The Higher Forms of Bacteria** (see end of Section II).—Some forms grow out into true or false branching threads and thus make a group of organisms intermediate between bacteria and the molds. These have been called higher bacteria or trichobacteria (see table, p. 26). They show increased complexity of structure and function: (1) in forming irregularly segmented filaments composed of elements similar to those found in the lower forms and showing either true or false branching; (2) in developing certain portions of their substance into reproductive bodies from which the new individuals grow (Plate II, Figs. 15 and 16).

The filaments seen sometimes among the lower forms have independent segments, which may easily separate and grow as tiny unicellular forms, while in the higher forms the filaments in their growth show a certain interdependence of their parts. For example, growth often occurs from only one end of the filament while the other becomes attached to some fixed object.

The members of the higher bacteria which are pathogenic for man have as yet been incompletely studied and classified. The following divisions serve as an attempt at differentiation:

1. *Leptothrix* grows in stiff, almost straight threads, in which division processes are seldom or never observed, and no branching has been seen.

2. *Cladothrix* grows in threads which rapidly fragment and produce false branching, that is, the terminal cell remains partly attached, but is pushed to one side by further growth from the parent thread; thus a Y-shaped growth is produced. Bacillary characteristics appear in old cultures.

3. *Actinomyces* grows in threads with true branching. Spores have been observed. It is characterized by the radiating wreath-like forms which it alone produces in the living body.

4. *Nocardia* (*Streptothrix*) grows in threads which produce abundant true branching; later there is fragmentation and formation of conidia.

*Reproduction among the Higher Bacteria.*—These forms increase in length for a time and then, at the free ends, or at intervals along the filaments, they produce small rounded cells, called conidia or spores, from which new individuals are formed. The terminal spores may be flagellated after their separation from the parent filament.

The flagellated forms may resemble certain flagellata among the protozoa.

### PROTOZOA.

**Definition.**—A protozoön (the lowest form of life classed in the animal kingdom) is a morphologically single-celled organism composed of protoplasm which is differentiated into cytoplasm and nucleus (or nuclear substance) both of which show many variations throughout the more or less complicated life cycle that each individual undergoes.

The protozoa are of higher grade than the bacteria because of their greater complexity in structure and life cycle (Plate IV).

Their shape and size vary so widely that no general description will fit all types. Some forms are small enough to pass through a Berkefeld filter, while the largest varieties described are about  $\frac{1}{4}$  inch long.

**The Cytoplasm.**—The cytoplasm consists of a mixture of substances, the most important of which belong to the proteins. It is more or less fluid, but, because of differences in the density and solubility of the several parts, it often presents an alveolar, linear, or granular appearance, which may come out clearly in fixed and stained specimens, but is usually not well seen in the living cells.

**Ectoplasm and Entoplasm.**—Frequently the protozoön cytoplasm is differentiated into a concentrated, viscid, more homogeneous, or hyaline outer layer called the *ectoplasm* and a more fluid granular central portion called the *entoplasm*. These two portions have different functions. The ectoplasm helps form the various organs (organelles) of motion, contraction, and prehension such as pseudopods (false feet), flagella (whip-like threads), cilia (hair filaments), suctorial tubules (through which food passes), and myonemes (contractile fibrils found in ciliates, gregarines, and a few flagellates). The entoplasm digests the food and contains the nucleus, as well as various granules and vacuoles. Some vacuoles serve as food digestors, and hence contain digestive ferments. The so-called contractile vacuoles which periodically fill and empty themselves may be considered as excretory organelles.

Other substances are seen from time to time in the entoplasm, such as bacteria, red blood cells, fatty granular pigments, bubbles of gas, crystals, etc.

**The Nucleus.**—The simplest morphological nucleus is a vesicular body which is differentiated from the cytoplasm by its essential constituent chromatin, so-called because it has a strong affinity for certain basic staining materials. Chromatin consists mostly of nuclein and appears in the form of smaller or larger granules, masses, or rods.

Generally, the chromatin particles are mixed with a second less intensely staining substance with more of an affinity for acid stains called plastin or paranuclein, similar to the substance from which the true nucleolus of the metazoön cell seems to be formed. This substance may appear in one or more distinct rounded bodies. Most of the chromatic substances of the nucleus in many protozoa are often massed together in an intensely staining ball-like body called the *karyosome* which undergoes various cyclic changes during the growth and development of the organism. The centrosome is generally

imbedded in the karyosome. The chromatin and plastin lie imbedded in a third substance in the form of an achromatic network called linin, which is closely related to the cytoplasmic network. There may or may not be a definite nuclear membrane. Sometimes there is no definitely structured nucleus, but the nuclear substance in the form of small chromatin masses or granules is distributed throughout the cytoplasm (the so-called "distributed nucleus") similar to that seen in bacteria.

*Somatic and Generative Chromatin.*—Some chromatin substances of the cell have physiological properties different from others. At times substances which have only vegetative properties are active, forming the so-called somatic or trophic chromatin; at other times, substances appear during sexual activities called generative or sexual or idiochromatin, and from these the vegetative (somatic) chromatin for the new cells is again formed. In the ciliata both these chromatin elements are present as distinct morphological bodies during the entire life of the organism, the somatic form in the macronucleus and the generative form in the micronucleus.

*Chromidia.*—The chromatin elements, in the form of granules, small irregular masses, threads, network, etc., which at certain stages pass from the nucleus into the cytoplasm, or which at times are, possibly, formed in the cytoplasm, were named "Chromidien" by R. Hertwig, who in 1899 first described their appearance. Their function in generative processes was demonstrated in 1903 by Schaudinn. During their formation the nucleus may entirely disappear, so that morphologically the cell may be considered non-nuclear. At a definite time thereafter new typical nuclei may be formed from those chromidial substances.

*Locomotor Nucleus (Kinetic Nucleus).*—In flagellates still another definite physiological chromatin is seen in the small body called the kinetic nucleus, which is either apart from or merged into a smaller body, the blepharoplast, forming the root of the flagellum. The kinetic nucleus is so called because it produces the locomotor apparatus. Both the kinetic and trophic nuclei may contain somatic and generative chromatin at the same time.

*The Centrosome.*—This is a small body which is always present in metazoan cells, playing an important part in cell division, but it has not been demonstrated as a morphological entity in many varieties of protozoa; part of the karyosome, however, may take its place, or there may always be a true centrosome within the karyosome. Whenever a centrosome appears in protozoa, it has its origin in the nucleus, resembling in this the kinetic nucleus and blepharoplast. All these four bodies (centrosome, blepharoplast, kinetic nucleus, and karyosome) therefore may be considered as having a similar morphological origin.

*Physiological Characteristics of Protozoa.*—In common with all other living organisms protozoa possess the characteristics of motility, nutrition, respiration, and reproduction.

*Motility.*—All protozoa react in certain characteristic ways toward chemical, mechanical, and electrical stimuli. Many are affected by light, while probably none reacts to sound. They manifest the reaction usually by motion of some sort. Most animal parasites, especially the higher forms, exert a positive taxis for leukocytes, principally for the large mononuclears and the eosinophiles. This fact is made use of in clinical diagnosis.

*Nutrition.*—Many protozoa, especially the pathogenic forms, absorb fluid food directly through the body wall; but the majority take in

solid food, such as small animal or vegetable organisms and organic waste, some through more or less definite regions of the body, others through any part of the surface by extending pseudopodia and entirely surrounding the food object, forming a so-called gastric vacuole.

After the food is digested the waste products are excreted, sometimes by osmosis, generally through special structures as the contractile vacuoles which regularly eject fluid substances to the outside of the organism.

**Respiration.**—It is supposed that the contractile vacuole has a respiratory as well as an excretory function. The interchange of gases is always going on, if not through a contractile vacuole, then by osmosis through any part of the wall.

**Growth and Reproduction.**—Under favorable conditions, new protoplasm is constructed rapidly, and the mass increases faster than the surface which, according to Spencer, initiates cell division. The changes generally appear first in the nucleus. The simplest variety of reproduction is a two-celled fission which may be either longitudinal or transverse, either of which may be direct (amitotic) or indirect (mitotic). A modification of equal fission is the so-called budding which may be single or multiple. When growth occurs so that fission is for a time incomplete, one cytoplasm containing several nuclei which finally separate into as many daughter organisms, the process is called multiplicative reproduction, or brood formation or internal budding. In the most extreme cases of multiplicative reproduction as it occurs among sporozoa the mother-cell with its nucleus separates simultaneously into large numbers of tiny daughter-cells. Such a process, when it occurs without conjugation and encystment, is called schizogony and the new cells are called merozoites. When such a multiplicative division occurs (generally after fertilization) within a cyst, it is spoken of as sporogony and the new cells are called sporozoites.

**Sexual Phenomena.**—Sexual phenomena (syngamy) fundamentally similar to those seen in metazoa have been observed in all groups of protozoa studied. The reproduction by the usual division or budding is interrupted at certain times in the life history of each organism and individuals come together in such a way that their nuclei fuse, usually after having undergone characteristic reduction divisions.

When the union is permanent, we speak of it as copulation and liken the process to that of the fecundation of the ovum by a spermatozoön. When the union is transient we call it conjugation. Here the two cells fuse for a time when the nuclei interchange protoplasm and then the cells separate and each one continues to grow and divide independently. When in a partly divided cell or in an apparently single cell, two nuclei, after undergoing reduction division, or its like, fuse, the process is called autogamy.

*The developmental cycle* of a protozoön consists of all the changes which occur in its growth from one act of fertilization to another (Plate IV, Fig. III). Many protozoa carry on the sexual part of their life cycle in one host and the asexual part in another (*e. g.*, malarial organisms).

**Cyst Formation.**—If protozoa do not get the required amount of water or air or suitable food, they cease their special movements, round out into more or less of a sphere and form a resisting membrane of chitin within which they may live for a long time, withstanding periods of desiccation, extreme heat and cold, and they may be blown about as dust until they find conditions again favorable for renewed growth; then water is absorbed, the cyst is ruptured and active life begins anew.

In parasitic forms encystment plays an important part in the passage from the old host to the new. The majority of forms would not be able to exist outside of the body of the host without having some protective membrane. The cyst may be formed simply for protection from drought, etc., when it is called a hypn cyst, from which the organism may emerge in about the same form as when it encysted; or the cyst may precede reproduction by spore formation or simple division, when it is called a sporocyst. In either case it may consist of a simple wall or it may be formed of several walls to enable it to resist prolonged desiccation, when it is called a resting cyst.

**Characteristics of Each of the Four Groups of Protozoa.—Flagellata.**—Flagellata are protozoa which move in the adult forms by one or several flagella or whip-like processes. If pseudopodia develop, they are transitory.

Generally the flagella arise from the anterior part of the organism, and in motion the larger ones (primary flagella) are directed forward, while smaller ones (secondary flagella) are directed backward, acting as rudders. Certain flagellata possess a modification of their bodies in what is called the undulating membrane, which consists of a fluted protoplasmic process attached along one side of the organism, the free edge of which is prolonged as the flagellum. It has been shown that flagella are not simple protoplasmic processes, but that they have more or less of a framework of elastic fibers as well, hence their power in locomotion can be better understood. Except with special stains, which bring out these fibers, they appear homogeneous.

The flagella arise from some definite place in the cytoplasm, sometimes from a distinctly differentiated chromatic body which has been given various names, such as blepharoplast, kinetic nucleus or centrosome, sometimes near this from a basal granule, microsome, diplosome, or flagellum root, sometimes directly from the nucleus. The basal granules seem to be derived primarily from the kinetonucleus, and may be considered from a physiological stand-point as a part of the motor nuclei.

The body of the flagellates is generally more or less elongated and, except in most primitive ones, is fixed in its outline. The latter characteristic is chiefly due to the fact that the organisms usually possess definite though delicate membranes containing elastic fibrils. The cytoplasm is usually not differentiated into an ento- and ectoplasm. It often contains one to several contractile vacuoles, as well as food vacuoles, and there is frequently a definite opening or cytostome for the entrance of food. There are usually many granules and inclusions



of various kinds scattered throughout the cytoplasm, and myoneme striations are seen in some forms. The nucleus, as a rule, situated anteriorly, varies much according to different species and to different stages of development.

The flagellata multiply either in the free motile condition or after encystment. In the first case, as a general thing, they divide longitudinally. The basal granules divide with the nuclei and the flagella

#### EXPLANATION OF PLATE IV.

Partly schematic. Rearranged and drawn by Williams. All stained by Giemsa.

##### I. FLAGELLATES.

Fig. 1.—Illustrating one flagellum. *Leishmania*: A, intracellular form; B, cultural forms.

Fig. 2.—Illustrating undulating membranes: A, *Trypanosoma lewisi*; B, *Trypanosoma brucei*; C, *Trypanosoma gambiense*.

Fig. 3.—Illustrating two flagella. *Bode lacertæ* (after Prowazek).

Fig. 4.—Illustrating four flagella. *Trichomonas*.

##### II. AMEBÆ.

Illustrating points considered differential in the two chief types of amebæ (entamebæ) described as parasitic in human beings.

Fig. 1.—*Entameba coli*, vegetative stage.

Fig. 2.—Dividing nucleus.

Fig. 3.—*Entameba coli* cyst containing eight nuclei.

Fig. 4.—*Entameba histolytica*, vegetative stage.

Fig. 5.—Four-nucleate cyst.

##### III. SPOROZOA.

A, description of Figs. 1 to 16. (After Schaudinn.) The life cycle of *Eimeria schubergi*.

In 1, the sporozoites, becoming free by bursting the sporocysts, pass out through an aperture in the wall of the oöcyst, and are ready to enter the epithelial cells of the host. 2 to 6 represent the asexual reproduction or schizogony, commencing with infection of an epithelial cell by a merozoite or a sporozoite; the merozoite after stage 6 may start again (5) at stage 2, as indicated by the arrows, or it may go on to the formation of gametocytes (9 to 11). 9 to 11 represent the sexual generation, the line of development becoming split into two lines—male (♂) and female (♀)—culminating in the highly differentiated gametes, which conjugate and become again a single line, shown in 12–14. The zygote thus formed goes on to the production of spores, 15 and 16. 2 and 3 represent epithelial cells showing penetration of a merozoite or a sporozoite and its change into a schizont; 4, the nucleus of the schizont divided into numerous daughter-nuclei; 6, segmentation of the schizont into numerous merozoites, about a central mass of residual protoplasm, which in this figure is hidden by the merozoites; 5, merozoites passing to reinfect host cell and repeat the process of schizogony; 7, 8, merozoites to be differentiated into male and female gametocytes; 9, the two gametocytes within a host cell; the microgametocyte (♂) has fine granulations; the macrogametocyte (♀) has coarse granulations. 11, a female gametocyte undergoing maturation; 13, mature macrogamete, freed from the host cell, and sending a cone of reception toward an approaching microgamete. In 12 the nuclei of the last stage have become microgametes, each with two flagella. The free microgametes are swimming to find a macrogamete. 14, the zygote (fertilized macrogamete), surrounded by a tough membrane or oöcyst, which allows no more microgametes to enter, and containing the female chromatin, which is taking the form of a spindle, and the male chromatin in a compact lump. 15, the nucleus of the zygote divided—the nuclei of the sporoblasts. In 16 the four sporoblasts become distinct, leaving a small quantity of residual protoplasm; each sporoblast has formed a membrane, the sporocyst. Within each sporocyst two sporozoites form about a sporal residuum.

B, *Babesia* infecting red blood cells: 1, pear-shaped bodies; 2, dividing forms; 3, eight pear-shaped bodies in a cell; 4, irregular ring-like bodies; 5, large, irregular body; 6, body with a flagellum-like projection.

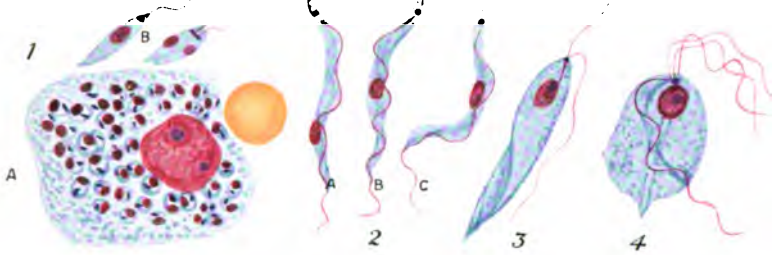
##### IV. CILIATES.

Fig. 1.—*Balantidium coli* (after Hartmann): A, adult form; B, C, dividing forms; D, conjugating forms,

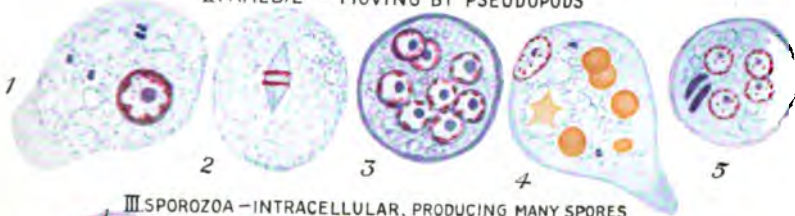
# PLATE IV

## TYPES OF PROTOZOA

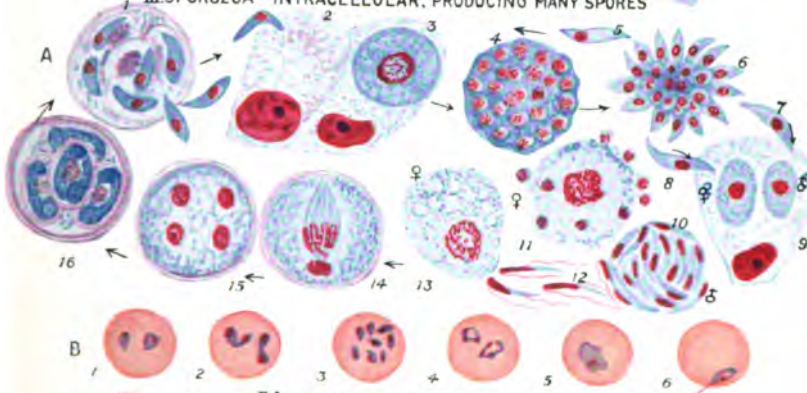
### I. FLAGELLATES—MOVING BY FLAGELLA



### II. AMEBÆ — MOVING BY PSEUDOPODS



### III. SPOROZOA—INTRACELLULAR, PRODUCING MANY SPORES



### IV. CILIATES—MOVING BY CILIA





of the daughter organisms are usually formed anew. Multiple division is also observed. In the second case the flagellata may or may not conjugate before they encyst. Then they divide within the cyst.

The sexual cycle varies much in different species. Isogamy has been noticed between fully grown individuals as well as between smaller forms. The union of different-sized forms, or anisogamy, has also been observed. Also autogamy is said not to be infrequent. It is claimed that certain of the flagellates pathogenic for man require a second host for the development of their sexual cycle.

The flagellates are subdivided into several orders, in only two of which are forms found which are pathogenic for man. (See Part II.) The different types of pathogenic flagellates are shown in Plate IV, 1, Figs. 1-4. Among the most important pathogenic forms are the trypanosomes.

**Trypanosoma.**—The name trypanosoma (boring animal) was given by Gruby, in 1843, to certain free-swimming hemoflagellates found by him in the blood of frogs. Much later similar flagellates were found in the blood plasma of many different species of vertebrates and in the intestinal tract of several blood-sucking invertebrates. Some of the forms, including those found in man, are pathogenic. A number of the blood-sucking insects are carriers of the diseased species to healthy animals.

Typical trypanosomes are characterized by a comparatively long, spirally twisted body, along one side of which is attached an undulating membrane having a cord-like edge that is continued forward as a free whip (flagellum). The flagellum arises near the posterior end of the organism in a small granule called the basal granule, which may be connected with the blepharoplast, a larger chromatin mass, called also the kinetonucleus because of its control over the motor apparatus. The nuclear apparatus consists of a macro- or trophonucleus, and of the above-mentioned kinetonucleus or blepharoplast, which last functions as a centrosome. The trophonucleus is usually situated near the middle of the organism; it is granular, thick, and egg-shaped, but varies somewhat in size and shape. The cytoplasm is faintly alveolar or granular, varying with age, environment, and possibly species. Toward the straight border of the cell the cytoplasm is more or less striated and in a few species definite myonemes are seen.

Reproduction occurs usually by longitudinal, occasionally by multiple division. The life cycle is not well known. Though transmission occurs through the bites of various invertebrates, notably flies, the few sexual changes described as taking place in the intestines of some of these intermediate hosts have not been fully corroborated. That an intermediate host is not necessary for the continued life of at least one species of trypanosome seems to be proved by the fact of direct transmission of *T. equiperdum* from horse to horse through coitus.

**Leishmania.**—Another important pathogenic flagellate is Leishmania. In humans this grows chiefly within large mononuclear cells. It shows its flagellated forms in cultures. (Plate IV, Fig. 1. See also Part II.)

**Amebida.**—Under amebida (syn., amebæ) we include forms composed of naked, simply constructed protoplasm having the power of producing lobose pseudopodia which are used as organs of motion and of nutrition.

The pseudopodia are protoplasmic processes which are projected in irregular succession from different parts of the surface of the cell, producing in this way an irregular motion. The form of the pseudopodia varies considerably in the different species. For instance, there are broad, blunt processes or narrow, less blunted ones, and each may be short or long, single or slightly branched. The cytoplasm may or may not take a share in their formation. The forms, of course, vary within limits according to the condition of the medium in which the amebæ are living. Movements are always called forth by some physical or chemical excitant. When such an excitant is desirable for food the pseudopods flow around it, and it is subsequently absorbed in the cytoplasm of the organism.

The members of this group may possess one nucleus or several. *Ameba binucleata* has two nuclei in the young adult stage, and *Pelomyxa palustris*, living in the bottom ooze of ponds, has an enormous number of nuclei. A marked feature of the nuclear apparatus is the formation of chromidia which, as has already been noted, may play such an important part in sexual reproduction. Generally each ameba has one contractile vacuole, but occasionally some are seen with several or with none.

Saprophytic forms belonging to this order are common. They may be found wherever there are moisture and decaying vegetable matter. The pathogenic forms are not so frequent. Because of the possibility that the still unknown causes of certain diseases (see Rabies and Smallpox) are organisms related to this order, it is especially important to study both saprophytic and pathogenic varieties, since a knowledge of the former which are more easily studied may help us understand obscure points in the life history of the latter.

Notwithstanding the common occurrence of saprophytic forms, the full life history of few of them has been worked out, and until the full cycle of development of any so-called ameba is known it is impossible to say whether that particular form belongs among rhizopoda or whether it is a developmental form of another group, as ameboid forms may occur at some time in the life history of all groups. It is quite possible that some of the organisms described as belonging to this order are really members of entirely different orders. For instance, it is known that the flagellate *Trichomonas* loses its flagella before copulation and crawls about by means of short blunt pseudopods as a typical ameba.

Amebæ reproduce by simple fission, by budding, and by brood formation. In the last case the reproduction is usually preceded by encystment. Two forms have been described most frequently as parasitic in man. The differences between them are shown in Plate IV, II, Figs. 1-5.

**The Sporozoa.**—The sporozoa are a group of exclusively parasitic protozoa of very wide-spread occurrence, living in the cells, tissues, and cavities of animals of every class. Generally they are harmless, but some varieties may produce pathological changes and even fatal diseases severely epidemic.

As their name indicates, they are all characterized by reproduction through spore formation, but they exhibit the utmost diversity of structural and developmental characteristics. As a rule, each species is parasitic on one kind of tissue of a particular species of host. They are generally taken into the system in the spore stage either (1) with the food of the host, (2) by the bites of insects, or (3) by inhalation. The spore membranes are dissolved by the fluids of the host, and thus one or more germs of sporozoites are set free to bore into the special cells of the host. Here they grow, some remaining permanently intracellular, others only in the young stages. The latter either pass different phases of their more or less complicated life history in different parts of the body of one and the same host or they pass some phases of their life cycle in the cells of an intermediate host.

The sporozoa vary widely in size as well as in other characteristics. From the smallest, several of which can be contained in a single blood cell, there are all gradations in size up to those that may be seen by the naked eye (*Parospora gigantea*, 16 mm.).

Besides being characterized by the power to produce very many resisting spores, the sporozoa are also characterized by the fact that as a class they possess none of the special organs found in other protozoa for ingesting or digesting solids. Many develop flagella during sexual phases or show ameboid movement during certain stages of their life cycle, but the flagella and pseudopodia are organs of locomotion and not of nutrition. Food vacuoles or contractile vacuoles have not been found. The life cycle of a typical sporozoön is rearranged and condensed from Schaudinn in Plate IV, III, Figs. 1-16.

**Ciliata.**—The ciliata (Plate IV) belong to the most complex of the protozoa. They possess a definite entoplasm containing nuclei and food vacuoles, and a definite ectoplasm containing basal granules from which arise the cilia which give the group its name. They have organoid structures which receive the food, some having definite mouth openings, and definite places for excreting waste products. The food vacuoles may contain acid or alkaline digestive products. The nuclear material is differentiated into two forms, a large macronucleus and a much smaller micronucleus. The function of the macronucleus is supposed to be vegetative, and that of the micronucleus reproductive. The macronucleus varies in size and shape and is completely filled with an alveolar chromatin. The micronucleus also varies in size and shape, and except in reproductive phases is generally vesicular in structure, with the chromatin heaped in one mass. Division of the nuclei takes place by mitosis in the case of micronuclei, and by amitosis, as a rule, in the case of the macronuclei. Under conditions unfavorable for growth the ciliata may encyst.

Conjugation is necessary to the life activity of these organisms. The phenomenon of conjugation in the ciliata has been well worked out. The micronuclei play the most important part, whereas the macronuclei simply break up and disappear in the protoplasm.

According to the arrangement of the cilia, the ciliata are divided into the four orders given in the general classification (see p. 26). Among these, the second, the order of the Heterotricha, interests us. In the Heterotricha the cilia are uniform over most of the body, while a specialized set fused into a series of firm vibratory plates is found about the mouth. Only one genus, *Balanidium*, has been observed in man (Plate IV; also Part II).

### CHEMICAL COMPOSITION OF MICROÖRGANISMS.

Quantitatively considered, the bodies of microörganisms consist largely of water, salts (chiefly phosphorus, potassium, chlorine, calcium iron and sulphur), fats, and albuminous substances. There are also present, in smaller quantities, extractive substances soluble in alcohol and in ether. Special varieties contain unusual substances, as wax and hemicellulose in tubercle bacilli. Each variety, furthermore, yields protein substances peculiar to itself, as shown in the effects produced by animal inoculation. At present we know but little concerning the differentiation of these specific substances. This subject will be taken up in detail under Toxins, etc. According to Cramer, many bacteria contain amyloid substances which give a blue reaction with iodine. True cellulose has not been found in bacteria by Vaughan or other workers, but large quantities of a gelatinous carbohydrate similar to hemicellulose have been obtained. Nuclein is found in all microörganisms. The nuclein bases—zanthin, guanin, and adenin—have been obtained in considerable amounts. Vaughan found no sodium chloride in his alcoholic extracts. There is a group of bacteria which contains large amounts of sulphur—viz., the *Beggiatoa*—and another group, the *Cladothrix*, is capable of separating ferric oxide from water containing iron.

Microörganisms possess the capacity to a high degree of accommodating their chemical composition to the variety of soil in which they are growing. Thus, *B. prodigiosus* when grown on potato contains 21.5 per cent. of dry residue and 2.7 per cent. of ash; when cultivated on turnips it contains 12.6 per cent. of dry residue and 1.3 per cent. of ash. Besides the concentration of the culture, its temperature and age also influence the amount of residue and ash produced. Qualitatively, a variation is shown by the addition of peptone in the culture media which tends to increase the percentage of nitrogenous matter in the microbe, or by the addition of glucose which decreases it.

The chemical composition of the bodies of animal parasites is an almost unexplored field. The ectoplasm and the cyst sacs in general are made up principally of a substance called chitin. Glycogen has

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been isolated from many forms. Proteolytic enzymes and acid secretion in digestive vacuoles have been demonstrated.

**Microchemical Reactions.**—To a certain degree the chemical composition of the *individual* organism may be studied both in the living and in the dead individual by the addition of the testing substances to a hanging drop or to a spread of such organism and the examination of it under the microscope. Thus, fats have been demonstrated by staining with osmic acid, Sudan III, or Scharlach R., as well as by alcohol-ether extraction.

Of special importance in this regard is the resistance which bacteria possess to diluted alkalis. Inasmuch as the majority of animal tissues are dissolved when treated with alkalis, this method has been adopted for rendering visible unstained bacteria in tissues. (See also Principles of Staining, p. 77).

## EFFECTS OF SURROUNDING FORCES UPON MICROÖRGANISMS.

1. **Food.**—Naturally, the effect of food upon organisms is marked. Though the majority of pathogenic microörganisms grow easily on certain artificial foods (culture media), some of them, like most of the protozoa, we have not yet been able to cultivate outside of the body of their host. Those microörganisms which seem to depend entirely upon a living host for their existence are known as *strict parasites*; those which live only upon dead organic (a few on inorganic) substances are called *strict saprophytes*; those which can lead a saprophytic existence, but which usually thrive only within living tissues, are called *facultative saprophytes*, while those that grow usually on dead material, but may grow within living tissues, are called *facultative parasites*. The *strict saprophytes*, which represent the large majority of all microörganisms are not only harmless to living organisms, but perform many exceedingly important functions in nature, such as the destruction of dead organic matter and its preparation for plant food through decomposition, putrefaction, and fermentation, while our group (see below, the Nitrifying Bacteria) are constructive in their activities. The *parasites*, on the contrary, may be harmful invaders (pathogenic microörganisms) of the body tissues, exciting by their growth and products many forms of disease. (See chapter on Relation of Microörganisms to Disease.) The substances essential for the majority of those forms which can be grown artificially are organic material as a source of carbon and nitrogen, an abundance of water, and certain salts. Either calcium or magnesium and sodium or potassium salts are usually required, also sulphur and phosphorus salts. Iron is demanded by a few varieties. The demands of microörganisms for food of a definite composition vary considerably. (See chapter on Cultivation of Microörganisms.)

While it is true that very wide differences in relative composition and total concentration of food media may have slight effect upon the general development of a given organism, slight changes in com-



position and reaction of the media often have a great effect upon morphology, rate of growth, motility, and specific products of growth.

**Reaction of Media.**—The reaction of the media is of very great importance. Most bacteria, particularly the pathogenic forms, grow best on those media that are slightly alkaline or neutral to litmus. Yeasts and molds grow best on a slightly acid medium. An amount of acid or alkali insufficient to prevent the development of bacteria may still suffice to rob them of some of their most important functions, such as the production of poison. The different effect upon closely allied varieties of bacteria of a slight excess of acid or alkali is sometimes made use of in separating those which may be closely allied in many other respects.

**Influence of One Species upon the Growth of Another.**—When one species of organism is grown in a food medium, that medium usually becomes less suitable for the growth of its kind and of other organisms. This is due partly to the impoverishment of the foodstuffs, but more to the production of chemical substances or enzymes. When different species are grown together, the antagonistic action of one upon the other may be shown from the beginning. Some species, however, have a coöperative or symbiotic action with other species.

In nature, microörganisms usually occur in mixed cultures (*e. g.*, in water, milk, intestinal contents of all animals), and here we may see antagonistic action in the prevalence of one species over others (*e. g.*, the lactic acid formers in the intestines), or coöperative action in the equal and luxuriant growth of two or more species (*e. g.*, pneumococcus and influenza bacillus in the lungs).

Experimentally, the existence of antagonisms can be demonstrated by inoculating alternate streak cultures of various bacteria on gelatin or agar media. It is found that many species will grow not at all or only sparingly when in close proximity to some other species. This antagonism, however, is often only one-sided in character. Again, when gelatin or agar plates are planted with a mixture of two species of bacteria, it may be observed that only one of the two grows. A third method of making this experiment is simultaneously to inoculate the same liquid medium with two species, and then to examine them later, both microscopically and by making plate cultures; not infrequently one species may take precedence over the other which after a time it may entirely overcome.

The symbiotic or coöperative action of microörganisms may be demonstrated experimentally in the following examples:

(a) Pneumococci, when grown together with a bacillus obtained from the throat, produce very large, succulent colonies. The influenza bacillus, which will not grow alone upon ordinary nutrient agar, will grow well there in the presence of certain other bacteria. Some anaërobic species grow even with the admission of air if only some aërobic species are present (tetanus bacilli with diphtheria bacilli).

(b) Certain chemical effects, as, for instance, the decomposition of nitrates, cannot be produced by many species of bacteria alone, but only when two are associated.

**2. Behavior toward Oxygen and other Gases.**—The majority of microörganisms absolutely require free oxygen for their growth, but a considerable minority fail to grow unless it is excluded. This latter fact, noted first by Pasteur, led him to divide germs into aërobic and anaërobic forms. Between these two groups we have those that can grow both with and without oxygen. Organisms that can grow under conditions other than the most favorable are called facultative organisms.

(a) **Aërobic Organisms.**—Growth only in the presence of free oxygen. The slightest restriction of air inhibits development. Spore formation, especially, requires the free admission of air.

(b) **Anaërobic Organisms.**—Growth and spore formation only on the total exclusion of free oxygen. Among this class of organisms are the bacillus of malignant edema, the tetanus bacillus, the bacillus of symptomatic anthrax, and many soil bacteria. Exposed to the action of oxygen, the vegetative forms of these bacteria are readily destroyed; the spores, on the contrary, are very resistant. Anaërobic germs being deprived of free oxygen—the chief source of energy used by the aërobic species to oxidize the nutritive substances in the culture media—are dependent for their oxygen upon decomposable substances, such as grape-sugar.

(c) **Facultative Anaërobic and Facultative Aërobic Organisms.**—The greater number of aërobic germs, including most of the pathogenic species, are capable of withstanding, without being seriously affected, some restriction in the amount of oxygen admitted (facultative anaërobes), and some grow equally luxuriantly under both conditions. Life in the animal body, for example, as in the intestines, necessitates existence with diminished supply of oxygen. If in any given variety of bacteria the amount of oxygen present is unfavorable, there will be more or less restriction in some of the life processes of this variety, such as pigment and toxin production, spore formation, etc. Pigment formation almost always ceases with the exclusion of oxygen, but poisonous products of decomposition may be more abundantly produced.

It has been observed not infrequently that certain species which on their isolation at first show more or less anaërobic development—that is, a preference to grow in the depth of an agar-stick culture, for instance—after awhile seem to become markedly aërobic, growing abundantly on the surface of the medium (facultative aërobes).

Those organisms that grow best or grow exclusively when the oxygen is only partly removed are called *micro-aërophilic organisms*.<sup>1</sup>

**Other Gases.**—While all facultative organisms as well as strict anaërobes grow well in nitrogen and hydrogen, they behave very differently toward carbonic acid gas. A large number of these species do not grow at all, being completely inhibited in their development until oxygen is again admitted—for example, *B. anthracis* and *B. subtilis* and other allied species. It has been found in some species, as glanders

<sup>1</sup> Lyon, in *Science*, 1917, lxx, 19, suggests that the word *oligaërobic* is better than the word *micro-aërophilic*.

and cholera, that the majority of the organisms are quickly killed by  $\text{CO}_2$ , while few, such as staphylococci, offer a great resistance, rendering impossible complete sterilization by means of this gas. The streptococcus as well as the staphylococcus exhibits a scanty growth. A mixture of one-fourth air to three-fourths carbonic acid gas seems to have no injurious effect on bacteria which cannot grow in an atmosphere of pure  $\text{CO}_2$ . Under pressure  $\text{CO}_2$  is more effective (page 58).

Sulphuretted hydrogen in large quantity is a strong bacterial poison. Even in small amounts it kills some bacteria.

**3. Effect of Temperature.**—Some form of microbic life is possible within the limits of  $0^\circ$  and  $70^\circ$  C. The maximum and minimum temperature for each individual species ordinarily lies from  $10^\circ$  to  $30^\circ$  C. apart, and the optimum covers about  $5^\circ$  C. Usually the temperature of the soil in which the germs are deposited is the controlling factor in deciding whether growth will or will not take place. Thus, nearly all parasitic microörganisms require for development a temperature near that of the body of their host, while many saprophytic forms grow best at temperatures lower than  $37^\circ$  C. Microbes when exposed to lower temperature than suffices for their growth, while having their activities decreased, may not be otherwise injured unless actually frozen for a certain time; when exposed to higher temperatures than allows of growth the life of the organism is more or less quickly destroyed. Sudden marked changes in temperature are detrimental.

Microörganisms have been classified according to the temperatures at which they develop, as follows:

*Psychrophilic Microbes.*—Minimum at  $0^\circ$  C., optimum at  $15^\circ$  to  $20^\circ$  C., maximum at about  $30^\circ$  C. To this class belong many of the water microörganisms, such as the phosphorescent bacteria in sea-water; and many molds and yeasts.

*Mesophilic Microbes.*—Minimum at  $5^\circ$  to  $25^\circ$  C., optimum about  $37^\circ$  C., maximum at about  $43^\circ$  C. To this class belong all pathogenic bacteria, most parasitic and many saprophytic forms.

*Thermophilic Microbes.*—Minimum at  $25^\circ$  to  $45^\circ$  C., optimum at  $50^\circ$  to  $55^\circ$  C., maximum at  $60^\circ$  to  $70^\circ$  C. This class includes a number of soil bacteria which are almost exclusively spore-bearing bacilli. They are also found widely distributed in feces.

By carefully elevating or reducing the temperature the limits within which a species will grow can be altered. Thus, the anthrax bacillus may be gradually made to accommodate itself to a temperature of  $42^\circ$  C., and pigeons, which are comparatively immune to anthrax, partly on account of their high body temperature ( $42^\circ$  C.), when inoculated with this anthrax succumb to the infection. Another culture accustomed to a temperature of  $12^\circ$  C. kills frogs kept at  $12^\circ$  C. We have cultivated a very virulent diphtheria bacillus so that it will grow at  $43^\circ$  C. and produce strong toxin.

**Effect of Low Temperature.**—Temperatures even far under  $0^\circ$  C. are only slowly injurious to microörganisms, different species being affected with varying rapidity. This has been demonstrated by numerous

experiments in which they have been exposed for weeks in a refrigerating mixture at  $-18^{\circ}\text{C}$ . If a culture of typhoid bacilli is frozen, about 50 to 70 per cent. of the organisms are killed at the time. At the end of one week not more than 10 per cent. survive, and at four weeks not over 1 per cent. After six months none survives. More resistant bacteria live longer and spores may survive in ice for years. Bacteria have even been subjected to a temperature of  $-175^{\circ}\text{C}$ . by immersing them in liquid air kept in an open tube for two hours, and 15 to 80 per cent. were found still to grow when placed in favorable conditions. We found about 10 per cent. of typhoid bacilli alive after thirty minutes' exposure to this low temperature. Staphylococci were more resistant. Spores were scarcely affected at all.

**Effect of High Temperatures.**—Prolonged temperatures from  $5^{\circ}$  to  $10^{\circ}\text{C}$ . over the optimum affect microörganisms injuriously in several respects. For instance, varieties may be produced of diminished activity of growth, the virulence and the property of causing fermentation may be decreased, and the power of spore formation may be gradually lost.

If the maximum temperature is exceeded, the organism dies. The thermal death-point for the psychrophilic species is about  $37^{\circ}\text{C}$ ., for the mesophilic species about  $45^{\circ}$  to  $55^{\circ}\text{C}$ ., and for the thermophilic species about  $75^{\circ}\text{C}$ . There are no non-spore-bearing bacteria, except possibly a few cocci, which when moist are able to withstand a temperature of  $100^{\circ}\text{C}$ . even for a few minutes. A long exposure to temperatures between  $60^{\circ}$  and  $80^{\circ}\text{C}$ . has the same result as a shorter one at the higher temperatures. Ten to thirty minutes' exposure to moist heat will at  $60^{\circ}\text{C}$ . kill the cholera spirillum, the streptococcus, the typhoid bacillus, and the gonococcus, and at  $70^{\circ}\text{C}$ . the staphylococcus, the latter being among the most resistant of the pathogenic organisms which show no spores. A much shorter exposure will kill a large percentage of any mass of these bacteria.

**Effect of Dry Heat.**—When microörganisms in a desiccated condition are exposed to the action of heated dry air, the temperature required for their destruction is much above that required when they are in a moist condition or when they are exposed to the action of hot water or steam. A large number of pathogenic and non-pathogenic species are able occasionally to resist a temperature of over  $100^{\circ}\text{C}$ . dry heat for from ten minutes to one hour. In any large number of bacteria a few are always more resistant than the majority. A temperature of  $120^{\circ}$  to  $130^{\circ}\text{C}$ . dry heat maintained for one and a half hours will destroy all bacteria in the absence of spores.

**Resistance of Spores to Heat.**—Spores possess a great power of resistance to both moist and dry heat. Dry heat is comparatively well borne, many bacterial spores resisting a temperature of over  $130^{\circ}\text{C}$ . for as long as three hours. Exposed to  $150^{\circ}\text{C}$ . for one hour, practically all spores are killed. Moist heat at a temperature of  $100^{\circ}\text{C}$ ., either boiling water or free-flowing steam, destroys the spores of most varieties of bacteria within fifteen minutes; certain pathogenic and

non-pathogenic species, however, resist this temperature for hours. The spores of a bacillus from the soil were destroyed after five and a half or six hours' exposure to streaming steam. They were destroyed, however, by exposure for twenty-five minutes in steam at 113° to 116° C. and in two minutes at 127° C. The spores from tetanus bacilli may require twenty minutes' exposure to kill them. Spores in fatty media are more resistant to heat.

The resistance of spores to moist heat is tested by suspending threads, upon which the spores have been dried, in boiling water or steam. The threads are removed from minute to minute and laid upon agar or in broth and kept at a suitable temperature for the germination of any living spores.

**4. Influence of Light.**—A large number of microörganisms are inhibited in growth by the action of bright daylight, more are affected by direct sunlight, and when the action of the sun's rays is prolonged they lose their power of developing when later placed in the dark.

Some motile organisms move toward the point of greatest luminosity, others away from it. Light-seeking protozoa have green or yellow chromatophores, and usually at the anterior end a red pigment spot. The violet and blue rays are more active than other parts of the spectrum in determining motion.

The susceptibility of bacteria to light may be tested, according to H. Buchner, by suspending a large number of bacteria in nutrient gelatin or agar and pouring the media while still fluid in Petri dishes, upon each of which has been pasted a strip of black paper on the side exposed to the light. The action of heat may be excluded by allowing the ray of light to pass through a layer of water or alum of several centimeters' thickness. After the plates have been exposed to the light for one-half, one, one and a half, two hours, etc., they are taken into a dark room and allowed to stand at 20° to 35° C. a sufficient length of time to allow of growth, and then examined to see whether there are colonies anywhere except on the parts covered by the paper; when the colonies exposed to the light have been completely destroyed, there is lying in a clear sterile field a sharply defined region of the shape of the paper strip crowded with colonies.

Protected by ordinary non-colored glass the sun's rays act very slowly.

Only the ultraviolet, violet, and blue rays of the spectrum seem to possess bactericidal action; green light has very much less; red and yellow light, none at all. The action of light is apparently assisted by the admission of air; anaërobic species, like the tetanus bacillus, and facultative anaërobic species, such as the colon bacillus, are able to withstand quite well the action of intense, direct sunlight in the absence of oxygen, for four hours.

According to Richardson and Dieudonné, the mechanism of the action of light may be at least partially explained by the fact that in agar plates exposed to light for a short time (even after ten minutes' exposure to direct sunlight) hydrogen peroxide ( $H_2O_2$ ) is formed. This is demonstrated by exposing an agar plate half-covered with

black paper, upon which a weak solution of iodide of starch is poured, and over this again a dilute solution of sulphate of iron; the side exposed to the light turns blue black. In gases containing no oxygen, hydrogen peroxide is not produced, and the light has no injurious effect. Access of oxygen also explains the effect which light produces on culture media which have been exposed to the action of sunlight, as standing in the sun for a time, when afterward used for inoculation. Some bacteria subsequently introduced into such media grow badly—far worse than in fresh culture media which are kept in the shade.

**Influence of Radium.**—*Radio-active fluids* have a slight inhibiting effect on microbial growth, but nothing decided enough to be used for therapeutic purposes has been evolved up to the present time.

**Influence of X-rays.**—These rays have a slight inhibiting effect on microörganisms when they are directly exposed to them.

**5. Influence of Electricity.**—The majority of the observations heretofore made on this subject would seem to indicate that there is no direct action of the galvanic current on bacteria; but the effect of heat and the electrolytic changes in the culture liquid resulting from the electrolysis may destroy them.

Protozoa may be contracted by moderate induction shocks and killed by strong ones.

When a current of electricity is passed through a liquid medium most active protozoa swim with their long diameters in the direction of the lines of force to assemble behind the cathode. Most flagellates and a few ciliates, however, move toward the anode. The direction of motion has been shown by Dale to vary with the nature and concentration of the medium. This whole question has been little studied.

**6. Influence of Agitation.**—Meltzer has shown that the vitality of bacteria is destroyed by protracted and violent shaking, which causes a disintegration of the cells. Many species are more quickly autolyzed after violent shaking. This fact is made use of in the production of bacterial vaccines.

**7. Influence of Pressure.**—Microörganisms in fluids which are subjected to great pressure are for a time inhibited in their growth. When oxygen or nitrogen are used, the same moderate inhibition occurs.

**Osmosis.**—Osmosis, due to differences of pressure between the medium and the microörganisms and to the permeability of the cell membrane for different substances is constantly occurring. Presumably the normal development of an organism takes place when the osmotic pressure within the cell is equal to (isotonic) that of its medium. When an organism is transferred to a new medium with an osmotic pressure markedly different from that of the old one, decided changes in morphology may occur. If the difference is too great or the transfer is too sudden, death may result. If the new medium has a higher pressure, then water is abstracted from the cell and the protoplasm shrinks from its membrane. This is called *plasmolysis*. When the new medium has a lower pressure than the old, the cell may burst. This process is called *plasmoptysis*.

**Influence of Carbonic Acid under Pressure.**—D'Arsonval and Charrin submitted a culture of *B. pyocyaneus* to a pressure of fifty atmospheres under carbonic acid. At the end of four hours cultures could still be obtained, but the bacillus had lost its power of pigment production. A few colonies were developed after six hours' exposure to this pressure, but after twenty-four hours no development occurred. Other bacteria subjected to pressure have exhibited more resistance. We have subjected broth and milk containing typhoid, dysentery, diphtheria, and colon bacilli to the gas under a pressure of 75 and 150 pounds. Within twenty-four hours 99 per cent. of those in the broth and 98 per cent. of those in the milk were destroyed. Within one week the broth was sterile and within four weeks the milk was sterile. Tubercle bacilli and staphylococci were much more resistant, but little effect was noticed in twenty-four hours. The results were the same whether the cultures were kept at 10° or 25° C. Bottled waters charged with carbonic acid are usually sterile.

**8. Effect of Drying.**—For growth, microorganisms require much moisture. Want of water affects them in different ways. Upon dried culture media development soon ceases; but on media dried gradually at the room temperature (nutrient agar, gelatin, potato) they live often for a long time, even when there are no spores to account for their longevity. A shrunk residue of such cultures placed in bouillon has often been found, after a year or more, to yield living bacteria. The yeasts and molds are still more resistant. The question as to how long the non-spore-bearing forms are capable of retaining their vitality when dried on a cover-glass or silk threads has been variously answered. We know now that there are many factors which influence the retention of vitality; spores, of course, are more resistant than vegetative forms.

The varying results sometimes reported by different observers may be explained by the fact that the conditions under which they were made were different, depending upon the desiccator used, the medium upon which the cultures were grown, and the use of silk threads or cover-glasses. In all these experiments, of course, it should be previously determined that in spore-bearing species there are no spores present. Even when a dried culture lives for a long time the majority of the organisms die in a few hours after drying. We have found 1,500,000 colon bacilli to be reduced to 100,000 after three hours' drying. In tissues or exudates they resist drying much longer than when unprotected.

Encysted protozoa withstand long periods of desiccation. Most forms when dried quickly remain viable much longer than when dried slowly.

**Duration of Life in Pure Water.**—When microorganisms which require much organic food for their development (and these include most of the pathogenic species) are placed in distilled water, they soon die—that is, within a few days. Their death is largely due to plasmoptysis. Even in sterilized well-water or surface water their life duration does

not usually exceed eight to fourteen days, and they rarely multiply. Instances, however, of much more extended life under certain conditions are recorded.

**9. Tactic Effect of Chemicals.—Chemotaxis.**—The deleterious effect of chemicals, especially those used as germicides, will be considered under Disinfection.

Some chemical substances exert a peculiar attraction for micro-organisms, known as *positive chemotaxis*, while others repel them—*negative chemotaxis*. Moreover, all varieties are not affected alike. Oxygen, for example, attracts aërobic and repels anaërobic bacteria, and for each variety there is a definite proportion of oxygen, which most strongly attracts. The chemotactic properties of substances are tested by pushing the open end of a fine capillary tube, filled with the substance to be tested, into the edge of a drop of fluid containing the organisms and examining under the microscope. We are able thus to watch the action of the microbes and note whether they crowd about the tube opening or are repelled from it. Among substances showing positive chemotaxis for nearly all microorganisms are peptone and urea, while among those showing negative chemotaxis are alcohol and many of the metallic salts. Such experiments are, of course, rough. The diffusion of the substances from the tube into the surrounding medium must play an extremely active role in the final result.

## PRODUCTS OF MICROBIAL GROWTH.

Microorganisms not only are acted upon by their surroundings, as has already been shown, but they themselves act, often markedly, upon these surroundings. We have spoken, under the Effect of Food (p. 51) of the great changes which may be produced in growths by slight changes in the food medium. So, many of the products, as noted below, are influenced to a greater or a less extent by environment.

**Production of Light.**—Microorganisms which have the property of emitting light (*photogens*) are quite widely distributed in nature, particularly in media rich in salt, as in sea-water. Many of these have been accurately studied. The emission of light is a property of the living protoplasm of the organism, and is not usually due to the oxidation of any photogenic substance given off by them; at least only in two instances has such substance been claimed to have been isolated. While these organisms cannot emit light except during life, they can live without emitting light. They are best grown under free access of oxygen in a culture medium prepared by boiling fish in sea-water (or water containing 3 per cent. sea-salt), to which 1 per cent. peptone, 1 per cent. glycerin, and 0.5 per cent. asparagin are added. The power of emitting light is soon lost unless the organism is frequently transplanted to fresh media.

**Thermic Effects.**—The production of heat by microorganisms does not attract attention in our usual cultures because of its slight amount, and even fermenting culture liquids with abundance of bacteria cause



no sensation of warmth when touched by the hand. Careful tests, however, show that heat is produced. The increase of temperature in organic substances when stored in a moist condition, as tobacco, hay, manure, etc., is due, partly at least, to the action of bacteria.

**Chemical Effects.**—The chemical changes which take place in substances as they are split up by microörganisms depend on the nature of the substances involved and the conditions under which they exist, and on the varieties of germs present. Chemists can as yet enumerate only some of the substances evolved, and describe, in but a few cases, the manner in which they were produced. The chemical activity may be divided into the following four types: (1) Production of substances which help in some way the life of the cell. These substances may be secreted and retained within the cell, or liberated from it, *e. g.*, ferments or enzymes; true toxins. (2) Production of substances liberated by the bacteria as waste products. (3) Production of substances by the breaking down of the food media, *e. g.*, putrefactive products, due largely to enzyme action. (4) The production of substances which help form the protoplasm of the bacterial cell itself.

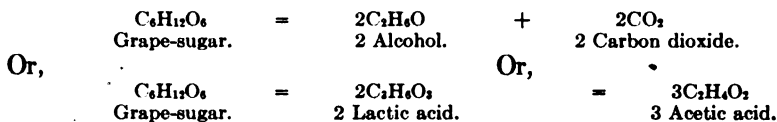
**Fermentation.**—Fermentation may be defined broadly as a chemical decomposition of an organic compound, induced by the life processes of living organisms (organized ferments or enzymes), or by chemical substances thrown off from the organisms (unorganized or chemical ferments or enzymes). It has been shown by Buchner and others that, in those cases of fermentation in which formerly it was believed the organized cell itself was necessarily concerned, ferments causing the same changes as the organized cells may be separated by various methods, such as crushing, filtering and so on. This brings fermentation by unorganized and organized ferments very closely together, the one being a substance thrown off from the cell, the other a substance ordinarily retained within the cell. The elaboration of both ceases with the death of the microörganisms producing them. Fermentation, therefore, requires the living agent or its enzyme. It furthermore demands the proper nutriment, temperature, moisture, and the absence of deleterious substances. The enzyme itself is not markedly diminished in quantity after the fermentation ceases, though the process yields products that inhibit its action; hence fermentation ceases when the products are in excess, or when the nutriment is exhausted. That the process will often begin again after diluting the fermented medium, shows that the *concentration* of the harmful products plays an important part in the inhibitory action. The fact that the enzyme apparently does not bind itself to any of the end-products allies it to those chemical agents known as "katalyzers."

**Characteristics of Ferments or Enzymes.**—Ferments are amorphous, non-dialyzable products of living protoplasm. They withstand moderate dry heat, but are usually destroyed in water solutions on exposure of ten to thirty minutes to a temperature of 60° to 70° C. They are injured by acids, but are resistant to all alkalis. They, even when present in the most minute quantities, are able partly to split up or

decompose complex organic compounds into simpler substances, and thus to render the foodstuff suitable for microbial growth.

The enzymes may be grouped as sugar-splitting, inverting, fat-splitting, proteolytic, diastatic, rennin-like or lab enzymes, and oxydizing enzymes.

*Sugar-splitting Enzymes (Zymase Lactase, Maltase, etc.).*—Many bacteria and yeasts are capable of splitting sugars, especially under anaërobic conditions. The action may be indicated by the following equations:



*Fat-splitting Enzymes (Lipase).*—Little is known about this enzyme. It has industrial importance because of its action in rendering butter and other fats rancid.

*The Proteolytic Enzymes.*—The proteolytic enzymes which are somewhat analogous to trypsin—being capable of changing albuminous bodies into soluble and diffusible substances—are very widely distributed. The liquefaction of gelatin, which is chemically allied to albumin, is due to the presence of a proteolytic ferment, gelase. The production of proteolytic ferments by different cultures of the same variety of bacteria varies considerably—far more than is generally supposed.

Bitter-tasting products of decomposition may be formed by certain liquefying bacteria in media containing protein, as, for example, in milk.

*Diastatic Enzymes.*—Diastatic ferments convert starch into sugar. This action is demonstrated by mixing starch paste with suitable cultures, then adding thymol and keeping the digestion for six to eight hours in the incubating oven; on the addition of Fehling's solution and heating, the reaction for sugar appears—the reddish-yellow precipitate due to the reduction of the copper.

*Inverting Enzymes.*—Inverting ferments (that is, those which convert polysaccharides into monosaccharides) are of very frequent occurrence. Bacterial invertase is slightly less susceptible to heat than are some others ferments, and is produced in culture media free from protein.

*Rennin-like or Lab Enzymes.*—Rennin-like ferments (substances having the power of coagulating milk and other liquid proteins with neutral reaction, independent of acids) are found not infrequently among bacteria.

*Oxydizing Enzymes.*—Oxydizing enzymes or oxydases produce change by the addition of oxygen. A common example of this is the production of vinegar chiefly by *B. aceti* and *B. pasteurianum*.

*Alkaline Products and the Fermentation of Urea.*—Aërobic bacteria always produce alkaline products from albuminous substances. Many species also produce acids from sugars, which explains the fact that

neutral or slightly alkaline broth often becomes acid at first from the fermentation of the sugar contained in the meat used for making the media. When the sugar is used up, the reaction often becomes alkaline, as the production of alkalis continues. The substances producing the alkalinity in cultures are chiefly ammonia, amine, and the ammonium bases.

The conversion of urea into carbonate of ammonia by *Micrococcus ureæ* affords an example of the production of alkaline substances by bacteria:



**Poisonous Products.**—The poisonous products of microörganisms are considered in Chapter VII.

**Pigment Production.**—Pigments have no known importance in connection with disease, but are of interest and have value in identifying bacteria. Very little is known of their chemical composition.

**Red and Yellow Pigments.**—Of the twenty-seven red and yellow chromogenic bacteria studied by Schneider, almost all produce pigments soluble in alcohol and insoluble in water. The large majority of these pigments possess in common the property of being colored blue green by sulphuric acid and red or orange by a solution of potash. Though varying considerably in their chemical composition and in their spectra, they may be classified, for the most part, among that large group of pigments common to both the animal and vegetable kingdoms known as *lipochromes*, and to which belong the pigments of fat, yolk of eggs, the carotin of carrots, turnips, etc.

**Violet Pigments.**—Certain bacteria produce violet pigments, also insoluble in water and soluble in alcohol, but insoluble in ether, benzol, and chloroform. These are colored yellow when treated in a dry state with sulphuric acid, and emerald green with potash solution.

**Blue Pigments.**—Blue pigments, such as the blue pyocyanin produced by *B. pyocyaneus*; and the fluorescent pigment common to many so-called fluorescent bacteria (bacteriofluorescence) are examples. In cultures the pigment is at first blue; later, as the cultures become alkaline, it is green.

Numerous investigations have been made to determine the cause of the variation in the chromogenic function of bacteria. All conditions which are unfavorable to the growth of the bacteria decrease the production of pigment, as cultivation in unsuitable media or at too low or too high a temperature, etc. The *B. prodigiosus* seldom makes pigment at 37° C., and when transplanted at this temperature, even into favorable media, the power of pigment production is gradually lost. *B. pyocyaneus* does not produce pigment under anaërobic conditions. Occasionally colored and uncolored colonies of the same species of bacteria may be seen to occur side by side in one plate culture, as, for example, in the case of *Staphylococcus pyogenes*.

**Reduction Processes.**—The following processes depend wholly or in part upon the reducing action of nascent hydrogen:

1. Sulphuretted hydrogen ( $H_2S$ ). All bacteria, according to Petri and Maassen, possess the power of forming sulphuretted hydrogen, particularly in liquid culture media containing much peptone (5 to 10 per cent.); only a few bacteria form  $H_2S$  in bouillon in the absence of peptone, while about 50 per cent. in media containing 1 per cent. peptone possess the property of converting sulphur into sulphuretted hydrogen, for which purpose is required the presence of nascent hydrogen. (For demonstration see Chapter IV.)

2. The reduction of blue litmus pigments, methylene blue, and indigo to colorless substances. The superficial layer of cultures in contact with the air shows often no reduction, only the deeper layers being affected. By agitation with access of air the colors may be again restored, but at the same time, if acid has been formed, the litmus pigment is turned red.

3. The reduction of nitrates to nitrites, ammonia, and free nitrogen. The first of these properties seems to pertain to a great many bacteria.

**Aromatic Products of Decomposition.**—Many microbes produce aromatic substances as the result of their growth. The best known of these are indol, skatol, phenol, and tyrosin. Systematic investigations have only been made with regard to the occurrence of indol and phenol.

**Decomposition of Fats.**—Pure melted butter is not a suitable culture medium for microbes. The rancidity of butter is brought about (1) as the result of a purely chemical decomposition of the butter by the oxygen of the air under the influence of sunlight, and (2) through the formation of lactic acid from the milk-sugar left in the butter. Fats are, however, attacked by bacteria with the consequent production of acid when mixed with gelatin and used as culture media.

**Putrefaction.**—By putrefaction is understood in common parlance every kind of decomposition due to microbes which results in the production of malodorous substances. Scientifically considered, putrefaction depends upon the decomposition of albuminous substances, which are frequently first peptonized and then further decomposed. Typical putrefaction occurs only when oxygen is absent or scanty; the free passage of air through a culture of putrefactive bacteria—an event which does not take place in natural putrefaction—very much modifies the process; first, biologically, as the anaërobic bacteria are inhibited, and then by the action of the oxygen on the products or by-products of the aërobic and facultative anaërobic bacteria.

As putrefactive products we have peptone, ammonia, and amines, leucin, tyrosin, and other amino substances; oxyfatty acids, indol, skatol, phenol, ptomains, toxins, and, finally, sulphuretted hydrogen, mercaptans, carbonic acid, hydrogen, and possibly marsh gas.

**Nitrifying Bacteria.**—According to recent observations, nitrification is produced by a special group of bacteria, cultivated in the laboratory with difficulty, which do not grow on our usual culture media. From the investigations of Winogradsky it would appear that there are two

common microörganisms present in the soil, one of which converts ammonia into nitrites and the other converts nitrites into nitrates.

**Conversion of Nitrous and Nitric Acids into Free Nitrogen.**—This process is performed by a number of bacteria.

The practical importance of these organisms is that by their action large quantities of nitrates in the soil, and especially in manure, may become lost as plant food by being converted into nitrogen.

By the aid of certain root bacteria, which gain entrance to the roots of legumes and there produce nodular formations, the leguminous plants are enabled to assimilate nitrogen from the atmosphere. It is not known exactly how this assimilation of nitrogen occurs, but it is assumed that the zoöglea-like bacteria, called *bacteroids*, constantly observed in the nodules, either alone or in a special degree, possess property of assimilating and combining nitrogen. It seems, moreover, to have been recently established that, independently of the assistance of the legumes, certain bacteria exist free in the soil, which accumulate nitrogen by absorbing it from the air. These various nitrifying, denitrifying, and nitrogen-fixing bacteria are described in detail in the special chapter upon bacteria in nature.

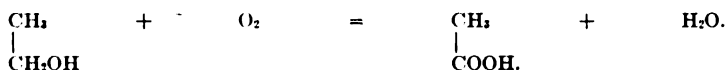
**Formation of Acids from Carbohydrates.**—Free acids are formed by many microbes in culture media containing some form of sugar or other fermentable carbohydrates, such as the alcohol mannite. The production of acid in ordinary bouillon takes place on account of the presence of sugar, which is usually found in small quantities in the meat.

If after the sugar is consumed, not enough acid has been formed to kill the bacteria, the acid is neutralized gradually and in the end the reaction becomes less acid or even alkaline.

Among the acids produced, the most important is lactic acid; also traces of formic acid, acetic acid, propionic acid, and butyric acid, and not infrequently some ethyl alcohol and aldehyde or acetone are formed. Occasionally no lactic acid is present.

**Formation of Gas from Carbohydrates and other Fermentable Substances of the Fatty Series.**—The only gas produced in *visible* quantity in sugar-free culture media is nitrogen. If sugar is vigorously decomposed by bacteria, as long as pure lactic acid or acetic acid is produced there may be no development of gas, as, for instance, with the *B. typhosus* on grape-sugar, but frequently there is much gas developed, especially in the absence of air. About one-third of the acid-producing species also develop gas abundantly, this consisting chiefly of CO<sub>2</sub>, which is always mixed with H. Marsh-gas is seldom formed by bacteria, with the exception of those decomposing cellulose. (For demonstration see Chapter IV.)

**Formation of Acids from Alcohol and other Organic Acids.**—It has long been known that *B. aceti* and allied bacteria convert dilute solutions of ethyl alcohol into acetic acid by oxidization:



The higher alcohols—glycerin, dulcitol, mannitol, etc.—are also converted into acids.

Finally, numerous results have been obtained from the conversion of the fatty acids and their salts into other fatty acids by bacteria.

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## CHAPTER III.

### THE MICROSCOPE AND THE MICROSCOPIC EXAMINATION OF MICROÖRGANISMS.

#### THE MICROSCOPE.

If lenses were capable of refracting all light equally, and bringing to a focus in one plane all rays proceeding from one plane in the object, the microscope would be a comparatively simple instrument. But simple lenses have several serious optical defects.

1. **Spherical Aberration.**—Points in the plane of the object are imaged on the *curved* surface of the spherical lens. This defect may be somewhat diminished by combining convex and concave lenses, and by restricting the size of the field. Objectives corrected in this way are called *aplanatic*.

2. **Chromatic Aberration.**—This defect is due to the fact that the rays of light vary in their refraction according to their wave length (colors), *e. g.*, the red rays have the longest focus and the violet the shortest. This is considerably corrected by combining planoconcave lenses of flint glass with biconvex lenses of crown glass—*achromatic objectives*.

Still more of a correction is made by combining several different kinds of lenses with a lens of fluorite—*apochromatic objectives*.

Monochromatic light may be employed and thus chromatic aberration may be entirely avoided.

3. **Diffraction.**—Less luminous secondary images about the primary image, due to scratches or foreign particles or other defects may occasionally occur in the very best lenses.

In order to understand fully the principle of the microscope, works on optics should be consulted.

**Different Parts of the Microscope** (Figs. 8 and 9).—A complete instrument usually has four oculars, or eye-pieces (*A*) which are numbered from 1 to 4, according to the amount of magnification which they yield. Nos. 2 and 4 are most useful for bacteriological work. The objective—the lens (*B*) at the distal end of the barrel—serves to give the main magnification of the object. For stained bacteria the  $\frac{1}{1\frac{1}{2}}$  achromatic oil-immersion lens is regularly employed; for photographic purposes the apochromatic lenses are needed, although even here they are not indispensable. A  $\frac{1}{1\frac{1}{8}}$  lens may at times be useful, but hardly necessary; a No. 4 ocular and a  $\frac{1}{1\frac{1}{2}}$  lens give a magnification of about 1000 diameters (Fig. 10). For unstained bacteria we employ either the  $\frac{1}{1\frac{1}{2}}$  immersion or  $\frac{1}{4}$  dry lens, according to the purpose for which we study the bacteria; for the examination of colonies when, as a rule, we do not wish to see individual bacteria but only the general

appearance of whole groups, we use lenses of much lower magnification (Fig. 11).

The stage (*C*)—the platform upon which the object rests—should be large enough to support the Petri plates if culture work is to be done. The distance between the optical axis of the instrument and the pillar must be great enough to permit one to examine rather more than half the surface of the Petri dish without revolving it. The iris diaphragm

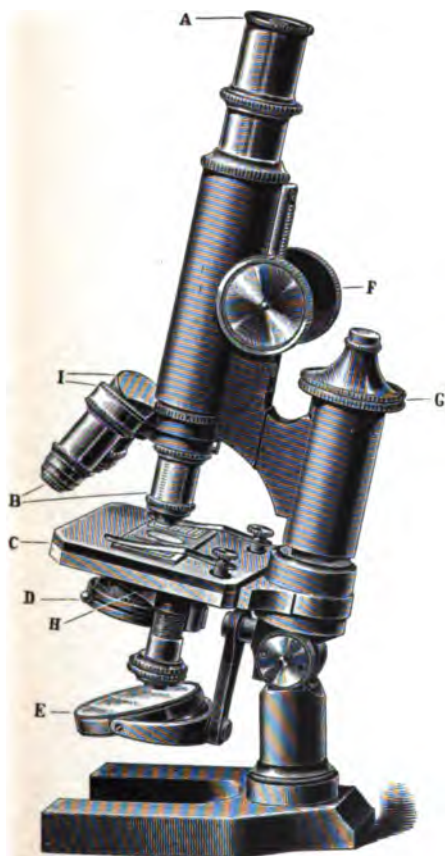


FIG. 8.—Microscope.

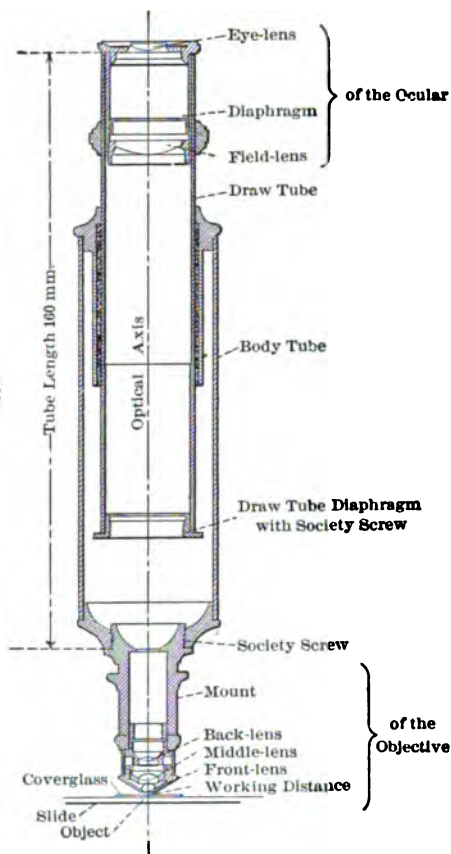


FIG. 9.—Internal structure of the microscope.

(*D*) opens and closes, and, like the iris of the eye, controls the amount of light. Its opening is diminished or increased by moving a small arm underneath the stage. The reflector or mirror (*E*) placed beneath the stage serves to direct the light to the object to be examined. It has two surfaces—one concave and one plane. The concave surface must not be employed when the substage condenser is being used, otherwise the rays of light reaching the stage from the condenser will not be correctly focused. The concave surface may be used when unstained



objects, such as colonies, or hanging drops are examined. At the same time the Abbé condenser should be lowered and the iris blender (*D*) regulated. The coarse adjustment (*F*) is the rack-and-pinion arrangement by which the barrel of the microscope can be quickly raised or lowered. It is used to bring the bacteria roughly into focus. If the bearings become loose, tighten the little screws at the back of the pinion box. Keep the teeth clean. If the bearings need oiling, use an acid-free lubricant, such as paraffin oil. The fine adjustment (*G*) serves to raise and lower the barrel very slowly and evenly, and is used for the exact study of the bacteria when high-power lenses are used. It is necessarily of limited range and delicate in its mechanism. If, when looking into the eye-piece, no change of focus is noticed by turning the micrometer head, or if the micrometer head ceases to turn, the adjustment has reached its limit. Raise the barrel of the microscope by means of the coarse adjustment, then turn the micrometer back to

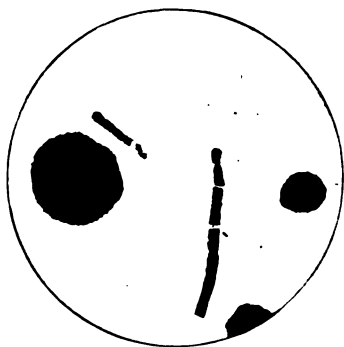


FIG. 10.—Anthrax bacilli and blood cells.  
× 1000 diameters.

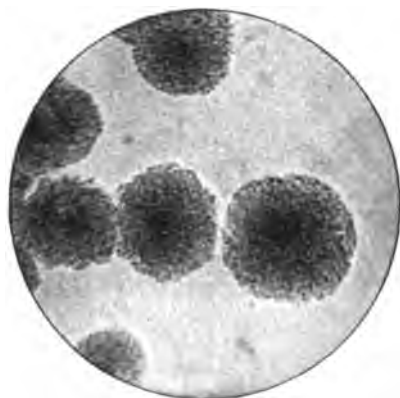


FIG. 11.—Colonies of diphtheria bacilli.  
× 200 diameters.

bring the fine adjustment midway within its range. When the fine adjustment head stops, do not force it. For the microscopic study of microbes it is essential that we magnify the organisms as much as possible and still have their definition clear and sharp. For this purpose the microscope should be provided with an oil-immersion system and a substage condensing apparatus. In using the oil-immersion lens a drop of oil (oil of cedar) of the same index of refraction as the glass is placed upon the face of the lens, to connect it with the cover-glass when the bacteria are in focus. There is thus no loss of light through deflection, as is the case in the dry system. If the lenses become dirty, they should be wiped gently with Japanese lens paper or a clean, soft, old-linen handkerchief. If necessary, breathe on the lens before wiping, and if this does not succeed, use a little xylol or chloroform. These substances are not to be used unless necessary. An immersion objective should always be cleaned immediately after

using. The objective should always be kept covered so as to prevent dust dropping in.

**Light.**—The best light is obtained from white clouds or a blue sky with a northern exposure. Avoid direct sunlight. If necessary, use white shades to modify the sunlight. Artificial light has one advantage over daylight in that it is constant in quality and quantity. The Welsbach burner and a whitened incandescent bulb give a good light. A blue glass between the artificial light and the lens is often of value. An eye shade may be helpful.

**Substage condensing apparatus** (*H*) is a system of lenses situated beneath the central opening of the stage. It serves to condense the light passing from the reflector to the object in such a way that it is focussed upon the object, thus furnishing the greatest amount of luminosity. Between the condenser and the reflector is placed the iris diaphragm.

**Focussing.**—Focus the body tube down by means of the coarse adjustment until the objective approaches very near to the cover-glass, being careful not to touch it. Then with the eye at the eye-piece focus up carefully with the coarse adjustment until the specimen comes plainly into view. Be careful not to pass this focal point. It is easily unnoticed if the light is too intense and the specimen thin and transparent. If the sliding tube coarse adjustment is used, focus carefully by giving the tube a spiral movement.

When the object is brought fairly well into focus by means of the coarse adjustment, use the fine adjustment to focus on the particular spot desired, for if this spot is in the centre of the field of the low power it should be somewhere in the field of the higher power. It is too much to ask of the maker that the lenses be made absolutely par-focal and centred. The delicacy of the centring can be appreciated when the magnification and the extremely small portion examined are considered. When the objectives are not thus fitted to the nose-piece, refocussing and again hunting up the object are necessary. In so doing we repeat the caution always to focus up before turning the nose-piece. When no revolving nose-piece is used, the change of objectives means the unscrewing of one and the screwing of the other into its place, and refocussing.

The beginner should always use the low-power objectives and oculars first. The low-power objectives have longer working distances and are not so apt to be injured. They always show a larger portion of the specimen and thus give one a better idea of the general contour. After obtaining this general idea the higher powers can be used to bring out greater detail in any particular part. Generally speaking, it is best to use a high-power objective and low-power eye-piece in preference to a low-power objective and high-power eye-piece. In the latter case any imperfections in the objective are magnified unduly by the eye-piece, giving, as a rule, poor definition.

**Tube Length and Cover-glass.**—All objectives are corrected to a certain tube length (160 mm. by most makers—Leitz, 170 mm.) and

all objectives in fixed amounts of over 0.7 N. A. are corrected to a definite thickness of cover-glass as well (Zeiss, 0.15 mm., 0.2 mm.; Leitz, 0.17 mm.; Bausch & Lomb and Spencer, 0.18 mm.). These objectives give their best results only when used with the cover-glass and tube length for which they are corrected. As indicated in Fig. 9 the tube length extends from the eye-lens of the eye-piece to the end of the tube into which the objective or nose-piece is screwed. If a nose-piece is used, the draw tube must be correspondingly shortened. If the cover-glass is thinner than that for which the objective is corrected, the tube must be lengthened to obtain best results; if thicker, shortened.

The more expensive objectives are provided with adjustable mounts by which the distances between the lens systems may be changed to compensate for difference of thickness of cover. They are successfully used only in the hands of an expert. One of them out of adjustment is worse than an ordinary objective.

**Dark-ground Illumination and the Examination of Ultramicroscopic Particles.**—The apparatus constructed by Siedentopf and Zsigmondy makes visible, even in solutions otherwise apparently homogeneous, very minute particles, which heretofore could not be seen even with the highest magnifications. Particles  $1\mu$  (a millimicron = one-millionth of a millimeter) are thus rendered visible.

This increased power in microscopic analysis is made possible by intense (electric arc lamp) lateral focal illumination of the objects examined, and by shutting off the rays passing in the usual way through the tube of the microscope. The greater the difference between the refractive index of the objects colloiddally dissolved or otherwise held in suspension and the fluid which surrounds them, the brighter will be the appearance of the objects, and therefore the more readily visible.

The microscopic field is dark; the objects which refract the light show as brightly illuminated, sharply defined pictures, in which the black margin corresponds to the contour of the object. The illuminated portion is surrounded by a fine dark zone, this in turn by alternate bright and dark zones, in which the illumination rapidly decreases.

Reichert, of Vienna, has recently simplified this apparatus by devising a new condenser. The light which illuminates the object has a greater refraction than the cone of light entering the objective which produces the image. Its advantages over the first method are: (1) It utilizes the source of light better; (2) any dry objective can be used without alterations; (3) small particles are seen without the disturbing refraction rings. With this apparatus such living organisms as the *Treponema pallidum*, and the flagella on certain bacteria, which can scarcely be seen by ordinary microscopes on account of their low refractive indices, may be demonstrated with great clearness.

The use of *microphotography with ultraviolet light* (according to A. Kohler) makes visible particles that cannot be seen by ordinary light, because of the inability of the violet rays to pass through certain substances, *e. g.*, chromatin.

This method is said to increase 40,000 times our present limit of vision. The few discoveries claimed by these means for diseases of unknown origin have so far lacked sufficient corroboration to constitute them proved.

**Double Microscopes.**—These have been devised by Metz and others for the purpose of making comparative studies of two objects.

### MICROSCOPIC METHODS.

The direct microscopic examination of suspected substances for micro-organisms can be made either with or without staining. Unstained, the microbes are examined living in a hanging drop or on transparent solid media, under daylight, or better, artificial light, to note their number, their motility, their size, form, and spore formation, their general arrangement, their reactions to specific serums and to vital stains, etc. But for more exact study of their structure they must be stained in a dried film preparation on a glass slide or a cover-glass or when in tissues, in sections.

**Elimination of Foreign Organisms from Preparations.**—Since germs are present in the air, in dust, in tap-water, on our bodies, clothes, and on all surrounding objects, it follows that when we begin to examine substances for microbes the first requisite is, that the materials we use, such as staining fluids, cover-glasses, etc., should be practically free from organisms, both living and dead, otherwise we may not be able to tell whether those we detect belong originally in the substances examined or only in the materials we have used in the investigation. Therefore, all solutions are filtered and all apparatus thoroughly cleaned and when necessary sterilized.

**Examination of Bacteria in the Hanging Drop.**—For this examination special slides and methods are desirable. The slide used is one in which there is ground out on one surface a hollow having a diameter of about  $\frac{1}{2}$  inch (Fig. 12). According to the purpose for which the hanging drop is to be studied, sterilization of the slide and cover-glass may or may not be necessary.



FIG. 12.—Hollow slide with cover-glass

The technic of preparing and studying the hanging drop is as follows: The surface of the glass around the hollow in the slide is smeared with a little vaselin or other inert oil. This has for its purpose both the sticking of the cover-glass to the slide and the prevention of evaporation in the drop placed in the little chamber, which is to be formed between the cover-glass when placed over the hollow, and the slide.

If the bacteria to be studied are in a fluid, we place a large platinum loopful upon the centre of the cover-glass and, to avoid drying, immediately invert it by means of a slender pair of forceps over the hollow in the slide, being very careful to have the drop over the centre of the cover-glass. The cover-glass is then pressed on the slide so as to spread the vaselin and make a perfect seal.

If the bacteria are growing on solid media, or are obtained from thick pus or tissues from organs, they are mixed with a suitable amount of bouillon or sterile physiological salt solution<sup>1</sup> either before or after being placed upon the cover-glass.

In studying living bacteria to determine only their grouping and motion we may use less magnification than for studying other characteristics. In studying unstained bacteria and tissues we shut off as large a portion of the light with our diaphragm as is compatible with distinct vision, and thus favor contrasts which appear as lights and shadows, due to the differences in light transmission of the different materials under examination. It is necessary to remember that they are seen with difficulty, and that we are very apt, unless extremely careful in focussing, to allow the lens to go too far, and so come upon the cover-glass, break it, destroy our preparation, and, if examining pathogenic bacteria, infect the lens. This may be avoided by first finding the hanging drop with a low-power lens and then centring it. The edge of the drop is focussed more easily than the centre. The lens of higher magnification is now very gradually lowered, while at the same time gently moving the slide back and forth the slightest extent possible with the left hand. If any resistance is felt, the lens should be raised, for it has gone beyond the point of focus and is touching the cover-glass.

**Hanging Mass or Hanging Block Cultures.**—In order to study the morphology and manner of multiplication of individual microörganisms to better advantage than in the hanging drop, we have used hanging masses of agar, made by placing a large platinum loopful of melted agar on a sterile cover-glass and allowing it to harden, protected from dust. The organisms are placed on the free surface of this mass which is then inverted over a hollow slide and studied as in a hanging drop.

Hill devised the following procedure: Melted nutrient agar is poured into a Petri dish to a depth of about  $\frac{1}{8}$  to  $\frac{1}{4}$  inch. When cool, a block is cut out about  $\frac{1}{4}$  inch square. The block is placed, under surface down, on a slide and protected from dust. A very dilute suspension of the growth to be examined is then made in sterile bouillon and spread over the upper surface of the block. The slide and block are then put in the incubator for ten minutes to dry slightly. A clean cover-slip is now placed on the agar block in such a way as to avoid large air bubbles. The slide is then removed. With the aid of a platinum loop a drop or two of melted agar is run along each side of the block to fill any angles between it and the cover-glass. After drying in the incubator for five minutes it is placed over a hollow slide and sealed with paraffin.

We consider the hanging-mass method better than that of the hanging block in many instances, because in the former method no pressure is exerted on the bacteria, and more oxygen is allowed them.

**Film Preparation** (spread, smear).—Film preparation is made as follows: A very small amount of the blood, pus, discharges from mucous membranes, cultures from fluid media, or other material to be examined is removed, usually by means of a sterile swab or platinum loop, and

<sup>1</sup> Physiological salt solution is usually 0.8 per cent. NaCl in distilled water.

smearcd undiluted in an even, thin film over a perfectly clean,<sup>1</sup> thin cover-glass or slide. From cultures on solid media, however, on account of the abundance of organisms in the material, a little of the growth is diluted by adding it to a small loopful of filtered or distilled water, which has been previously placed on the glass slide. It is best to add to the drop just enough of the culture to make a perceptible cloudiness. Blood films may be made either by the cover-glass or the slide method.

To make a cover-glass preparation, two square, very thin (hence flexible) cover-glasses are cleaned. Holding one with thumb and index fingers by opposite corners, the tip of a drop of blood obtained by needle puncture of finger or lobe of ear is made to touch the centre of the cover-glass, and the second clean cover-glass held similarly is allowed to fall upon the first one in such a manner that the corners do not coincide. The blood droplet spreads by capillarity into a thin film, which is a sign to pull the two covers apart in the plane in which they lie; good results depend upon cleanliness, rapidity, and success in sliding the two covers apart.

To make a slide film, the tip of the exuded blood drop is made to touch one slide near one end, and the edge of the second slide, held at an acute angle to the first one, is made to bisect the drop, which will spread at the point of contact by capillarity across the slide. Upon pulling the second or spreading slide over the first slide, never changing the angle and applying gentle pressure, a thin layer of blood suitable for examination will be formed. A slide made in this manner should be dried immediately by agitation in the air. It may then be fixed and stained in various ways.

Milk films, after fixation, are cleared of fat by means of ether, xylol or alkaline solution.<sup>2</sup>

The film either is dried thoroughly in the air and then fixed with heat or any chemical fixative, or it may be placed in any of the fixatives while still moist. The usual fixatives are methyl alcohol, absolute ethyl alcohol, Zenker's solution, etc. (see p. 85).

When fixed with heat, the glass is held by any one of the several kinds of forceps commonly used, and is passed three times by a rather slow movement through the Bunsen or alcohol flame.

The film thus prepared is usually stained either by the simple addition of a solution of an aniline dye, for from a few seconds to five minutes,

<sup>1</sup> To render new cover-slips clean and free from grease, the method recommended by Gage is useful: Place in following solution over night:

Bichromate of potash ( $K_2Cr_2O_7$ ) . . . . .	200 grams
Water, tap or distilled . . . . .	800 c.c.
Sulphuric acid . . . . .	1200 c.c.

The bichromate is dissolved in the water by heating in an agate kettle; the sulphuric acid is added very slowly and carefully on account of great heat developed. After cooling, it is kept in a glass vessel. It may be used more than once.

Glasses are removed the next morning and cleansed in running tap-water until the yellow color disappears. They are then placed in ammonia alcohol until used. When used, wipe with soft, clean linen or cotton cloth. If old cover-slips are used, boil first in 5 per cent. sodium carbonate solution.

Another procedure is, after washing with soap and water and rinsing in water, to soak the cover-glasses in alcohol, then wipe with soft linen, then place in a Petri dish, and heat in the dry steriliser for one hour at  $200^\circ$  C. to burn off fatty substances. The heating may be done by holding the cover-glass in the flame sufficiently to heat thoroughly without softening. A cover-glass is not clean when a drop of water spread over it does not remain evenly distributed, but gathers in droplets.

<sup>2</sup> One-half or 1 per cent. sodium hydrate.

or by one of the more complicated special stains described later. When the stain is to be hastened or made more intense, the dye is used warm.

The cover-glass or slide, with the charged side uppermost, may either rest on the table or be held by some modification of Cornet's forceps. When the solution is to be warmed, the cover-glass may be floated, smeared side down, upon the fluid contained in a porcelain dish resting on a wire mat, supported on a stand; or the solution may be poured on the glass which may then be held over the flame in the Cornet forceps. If a slide is used, it is simply inserted in the fluid or covered by it. The fluid both in the dish and on the slide should be carefully warmed so as to steam without actually boiling. The slide should be kept completely covered with fluid.

After staining the film, the cover-glass or slide is grasped in the forceps and thoroughly but gently washed in clean water and then dried, first between layers of filter paper and then in the air or high over a flame. If a cover-glass has been used, a drop of balsam or water is placed on a glass slide and the cover-glass put upon it with the film side down. Films made on slides are usually unmounted. Cedar oil is added at the time of examination with the oil-immersion lens and washed off with xylol immediately after.

*Burri's India-ink Method of Demonstrating Bacteria.*—In 1907, 1908, and 1909 Burri recommended the following method for isolating and studying single bacterial cells. A solution of India ink (flüssige Perltusche) in water 1 to 10 (better 1 to 4) is sterilized in test-tubes in the autoclave for fifteen minutes. A small drop of this ink is mixed carefully with a drop of the fluid to be examined. If cultures from isolated cells are desired, the fluid should first be diluted so that a drop contains *presumably* a single organism; then drops of the mixture are placed in rows upon nutrient agar plates. If the bacteria are to be examined immediately, a drop of the mixture (ink plus undiluted bacterial fluid) is allowed to dry upon a glass slide and then examined under an oil-immersion lens. The bacteria appear a brilliant white upon a dark field, particles of the ink surrounding the organisms like a capsule. This method is especially applicable for the demonstration of such organisms as the *Tr. pallidum* which have poor staining qualities and a low index of refraction.

**Stains and Staining Methods for Microörganisms.**—Protozoa stain in general as do animal cells. The protoplasm of mature bacteria reacts to stains much as does the nuclear chromatin of animal cells, though the intensity of the staining varies somewhat with the condition of growth, such as the age, the species, the media, and so on.

The best bacterial stains are the basic aniline dyes, which are compounds derived from the coal-tar product aniline ( $C_6H_5NH_2$ ).<sup>1</sup>

**Aniline Dyes.**—The aniline dyes which are employed for staining purposes are divided into two groups. In one the basic part of the molecule acts and the stains are spoken of as nuclear stains, since they color the nuclear chromatin of both cells and bacteria. In the other

<sup>1</sup> For a good description of the composition and action of the various stains, see A. B. Lee's *Microtometist's Vade-Mecum*, 7th edition, 1913.

the staining act depends upon the acid part of the molecule, and the bacteria and cytoplasm of the higher cells are stained faintly. The stains in the latter group are used chiefly for contrast coloring. The basic dyes are usually employed as salts of hydrochloric acid, while the acid dyes occur as sodium or potassium salts.

The following are the most commonly used basic aniline stains:

Blue stains—methylene blue, thionin blue } (give the best differentiation;  
Red stains—basic fuchsin, safranin } difficult to overstain).

Brown stain—Bismarck brown (weak, may be used as counter-stain).

Green stain—methyl green.

Pink stain—eosin (weak; may be used as counter-stain).

Violet stains—methyl violet, gentian violet, crystal violet (most intense stains; may overstain).

These dyes are all more or less crystalline powders, and while some are definite chemical compounds, others are mixtures. For this reason various brands are met with on the market, and the exact duplication of stains is not always possible. Dyes should be obtained from reliable houses only.

It is advisable to keep on hand *stock saturated alcoholic solutions* from which the staining solutions are made. These stock solutions are made by pouring into a bottle enough of the dye in substance to fill it to about one-quarter of its capacity. The bottle should then be filled with 95 per cent. ethyl alcohol, tightly corked, well shaken, and allowed to stand twenty-four hours. If at the end of this time all the staining material has been dissolved, more should be added, the bottle being again shaken and allowed to stand for another twenty-four hours. This must be repeated until a permanent sediment of undissolved coloring matter is seen upon the bottom of the bottle. This bottle will then be labelled "saturated alcoholic solution," of whatever dye has been employed. The dilution for use in staining is made by filling a small bottle three-fourths with distilled water, and then adding the concentrated alcoholic solution of the dye, little by little, until one can just see through the solution. It is sometimes desirable to use a more concentrated solution with dyes such as methylene blue. Care must be taken that the color does not become too dense; usually about 1 part to 10 is sufficient.

#### General Observations on the Principles of Staining Bacteria.—

The staining of bacteria is not to be considered simply as a mechanical saturation of the cell body with the dye, in which the latter is dissolved in the plasma. It is rather a chemical combination between the dye substance and the plasma.

The dependence of the staining process upon the solvent condition of the dye is shown in the following observations:

1. Entirely water-free, pure alcoholic dye solutions do not stain well.
2. Absolute alcohol does not decolorize bacteria, while diluted alcohol is an active decolorizing agent. The compound of dye substance and plasma is therefore insoluble in pure alcohol.
3. The more completely a dye is dissolved the weaker is its staining power. For this reason pure alcoholic solutions are inactive; and the so-called weak dye solutions to which strong dye solvents have been added are limited in their action on certain bacteria in which the dye substance is closely united. This is the principle of Neisser's stain for diphtheria bacilli, viz., acetic-acid-methylene-blue solution.

On the other hand, the addition of alkalis to the dye mixture renders the solvent action less complete and the staining power more intense. According to Michaels, however, in Löffler's methylene-blue solution the role of the alkali is purely of a chemical nature, by which it converts the methylene blue into methylene azure (azure II).



The dependence of the staining process upon the nature of the microbe is exhibited in the following facts:

Certain microbes stain easily, others with difficulty. To the latter belong, for example, the tubercle bacillus and lepra bacillus. Spores and flagella also stain with difficulty. The easily stained objects require but a minimum of time to be immersed in a watery solution, while the others must be stained by special dyes with or without the aid of outside influences (heat, mordants, etc.). The difficultly stained objects are at the same time not easily decolorized. The explanation of the resistance which these bacteria show to staining as well as to decolorizing agents is to be sought in two ways: either on the assumption that they possess a difficultly permeable or a resisting envelope, or that they have a special chemical constitution. The latter hypothesis holds good only, if at all, in regard to flagella and spores; while the assumption of the resisting envelope has reference more particularly to the tubercle bacillus, and is probably correct. The presence of fatty and waxy bodies in the envelope of these microörganisms is capable of demonstration. Moreover, after extraction of these bodies by ether the tubercle bacillus loses its power of resisting acids, which peculiar resistance can also be artificially produced in other bacteria having normally no such resisting power. In many instances, doubtless, both of these causes, viz., resistant envelope and chemically different constitution, work together to produce the above-mentioned results.

Elective staining properties, whereby certain species of organisms are exclusively or rapidly and intensely stained by certain dyes, have repeatedly been observed. Of the greatest practical importance in this respect is the *Gram stain* (see p. 78, and Chapter VI), used for the differential diagnosis of many species of bacteria; although a distinct classification of bacteria into those which are stained and those which are not stained by Gram's solution has been shown to be impracticable. There are some bacteria, however, which act uniformly toward Gram under most conditions; as, for example, the anthrax bacillus and the pyogenic cocci are always positive, the cholera and plague bacilli and gonococci are always negative to Gram. Other species, again, are at one time stained and at another decolorized by Gram; thus pyocyanus is stained only in young individuals. Previous heating or extraction with ether does not prevent the action of Gram's stain, but treatment with acids or alkalies renders it impossible. Bacteria so treated, however, after one hour's immersion in Löffler's mordant regain their property of staining with Gram.

As to the nature of Gram's staining solution, it may be mentioned that only the pararosanilines (gentian violet, methyl violet, and Victoria blue) are suitable for the purpose, whereas the rosanilines (fuchsin and methylene blue) give negative results. The reason for this is that the iodine compounds with the pararosanilines are fast colors, while those with the rosanilines are unstable. These latter compounds when treated with alcohol break up into their constituents, the iodine is washed out, and the dye substance remaining in the tissues stain them uniformly; that is, without differentiation. But iodine-pararosaniline compounds are not thus broken up and consequently stain those portions of the tissue more or less, according to the affinity which they have for the dye substance. The parts stained by Gram are thus distinguished from those stained violet, not only quantitatively, but qualitatively; it is not a gentian violet, but an iodine-pararosaniline staining which occurs.

**Mordants and Decolorizing Agents.**—We have already noted that the protoplasm of unrelated microbes may respond differently to the several dyes. There is, however, seldom any difficulty in selecting a dye which will stain sufficiently to make microbial cells in pure cultures distinctly visible. When the microbes are imbedded in tissue or mixed in a film with blood or pus, it is frequently difficult to prevent the stain from so acting on the tissue or pus elements as to obscure the organisms.

Various methods are then employed to stain the germs more intensely than the tissues or to decolorize the tissue more than the organisms. Heating, the addition of alkali to the staining fluid and prolonging the action of the dyes increase the staining properties. We regulate these so as to give the best results. We also use mordants; that is, substances which fix the dye to the bacterial cell, such as aniline oil or solutions of carbolic acid on metallic salts. The decolorizing agents used chiefly are mineral acids, vegetable acids, diluted alcohols, various oils, and hot water.

**Formulae of the More Generally Used Staining Mixtures.**—**LÖFFLER'S METHYLENE-BLUE SOLUTION.**—Saturated alcoholic solution of methylene blue, 30 c.c.; caustic potash in a 0.01 per cent. solution, 100 c.c. (The alkali not only makes the cell more permeable, but also increases the staining power by liberating the free base from the dye.)

Films stained two to five minutes, heated if more intense stain is desired. Sections stained one-quarter to several hours and decolorized until faint blue; contrast stain eosin; washed, dehydrated, cleared, and mounted (see p. 85).

**CARBOL-FUCHSIN, OR ZIEHL-NEELSEN SOLUTION.**—Distilled water, 100 c.c.; carbolic acid (crystalline), 5 gm.; alcohol, 10 c.c.; fuchsin, 1 gm.; or it may be prepared by adding to 100 c.c. of a 5 per cent. watery solution of carbolic acid, 10 c.c. of a saturated alcoholic solution of fuchsin. The carbolic acid, like the alkali, favors the penetration of the stain.

The last two methods, combined with heating, are used to stain spores and certain resistant bacteria as the tubercle bacilli and other "acid resisters," so that they retain their color when exposed to decolorizing agents (see below).

**Carbol-gentian Violet.**—One part of saturated alcoholic solution of gentian violet to 10 parts of a 2 to 5 per cent. solution of carbolic acid.

**Carbol-methylene blue**, first used by Kühne, consists of 1.5 gm. of methylene blue, 10 gm. of absolute alcohol, and 100 c.c. of a 5 per cent. solution of carbolic acid. **Carbol-thionin** consists of 10 parts of a saturated alcoholic solution of thionin and 100 parts of a 1 per cent. solution of carbolic acid.

**POLYCHROME METHYLENE BLUE (GOLDHORN).**—To prepare the stain dissolve 1 gm. lithium carbonate in 200 c.c. clean water and add 1 gm. methylene blue. Shake and dissolve. Pour into porcelain dish over water-bath, stirring frequently until blue color changes to a rich purple. Run through cotton in funnel; make up to 200 c.c. To 100 c.c. add 5 per cent. acetic acid until a faint pink is just visible on litmus paper above level of point discolored by the dye. Now add the remaining 100 c.c. of dye and allow to stand in open dish for forty-eight hours. Run once more through cotton into clean bottle.

It is not necessary to use distilled water, and satisfactory results are obtained with all the different forms of methylene blue tried. B-X (Grübler) is preferable.

Fix the smear by immersion in commercial wood alcohol for fifteen to thirty seconds; wash well and stain for about ten to fifteen seconds in polychrome; wash and stain for from fifteen to sixty seconds in 0.05 per cent. aqueous eosin. Wash again in water and dry in air without heat. Body of parasites blue; chromatin is red to purple.

Results may be varied by using polychrome or eosin for different lengths of time. Admirable preparations may be obtained, even when there is precipitation, by just rinsing the smear a little in 50 per cent. ethyl alcohol. This will remove any precipitation.

**KOCH-EHRlich ANILINE WATER SOLUTION OF FUCHSIN OR GENTIAN VIOLET** is prepared as follows: To 98 c.c. of distilled water add 2 c.c. aniline oil, or, more roughly but with equally good results, pour a few cubic centimeters of saturated aniline oil into a test-tube, then add sufficient water nearly to fill it. In either case the mixtures are thoroughly shaken and then filtered into a

beaker through moistened filter paper until the filtrate is perfectly clear. To 75 c.c. of the filtrate (aniline water) add 25 c.c. of the saturated alcoholic solution of either fuchsin, methylene blue, or gentian violet, or add the alcoholic solution until the aniline water becomes opaque and a film begins to form on the surface.

**GRAM'S STAIN.**—Another differential method of staining which is employed is that known as Gram's method. In this method the object to be stained is floated on or covered with the aniline or carbolic gentian-violet solution described above. After remaining in this for a few minutes it is rinsed in water and then immersed in an iodine solution (Lugol's), composed of iodine, 1 gm.; potassium iodide, 2 gm.; distilled water, 300 c.c. In this it remains for from one to three minutes and is again rinsed in water. It is then placed in strong alcohol until most of the dye has been washed out. If the cover-glass as a whole still shows a violet color, it is again treated with the iodine solution, followed by alcohol, and this is continued until no trace of violet color is visible to the naked eye. It may then be washed in water and examined, or before examination it may be counter-stained for a few minutes by a weak solution of a contrasting dye, such as eosin, fuchsin, carmine, or Bismarck brown. This method is useful in demonstrating the capsule which is seen to surround some bacteria—particularly the pneumococcus—and also in differentiating between varieties of bacteria; for some do and others do not retain their stain when put in the iodine solution for a suitable time (see Chapter VI, for further remarks upon Gram's stain; see also p. 76).

*The Modifications of Gram's Stain are Many.*—One only is given here.

**Nicoll's Modification.**—Stain cold in carbol-gentian violet one minute; wash in tap-water; stain cold in the iodine mixture one minute; wash in tap-water; decolorize ten seconds in acetone (1 part) and alcohol (3 parts); wash in tap-water; counter-stain ten seconds in dilute carbol-fuchsin (1 to 10).

**Stain for *B. Diphtheriæ*. Neisser Stain.**—The Neisser stain is carried out by placing the cover-slip smear of diphtheria or other bacilli in solution No. 1 for from two to three seconds, and then, after washing, in No. 2 for from three to five seconds. The bacilli will then appear either entirely brown or will show at one or both ends a dark blue, round body. With characteristic diphtheria bacilli, taken from a twelve to eighteen hours' growth on serum, nearly all will show the blue bodies (Fig. 116), while with pseudo types (Fig. 122), few will be seen.

The solutions are as follows:

No. 1.	
Alcohol (96 per cent.) . . . . .	20 parts.
Methylene blue (Grübler) . . . . .	1 part.
Distilled water . . . . .	950 parts.
Acetic acid (glacial) . . . . .	50 parts.

No. 2.	
Bismarck brown . . . . .	1 part.
Boiling distilled water . . . . .	500 parts.

**Stain for *B. Pertussis*.**—Toluidin blue, 5 grams; alcohol, 100 c.c.; water, 500 c.c.

**Staining of Capsules.**—Many methods of demonstrating the capsule have been devised. Three only will be given here.

**WELCH'S GLACIAL ACETIC ACID METHOD** is as follows: (1) Cover the preparation with glacial acetic acid for a few seconds; (2) drain off and replace with aniline gentian-violet solution; this is to be repeatedly added until all the acid is replaced; (3) wash in 1 or 2 per cent. solution of sodium chloride and mount in the same. Do not use water at any stage. The capsule stains a pale violet. (See Plate III, Fig. 15.)

**HISS'S COPPER SULPHATE METHOD** (Fig. 13).—The organisms are grown, if possible, on ascitic fluid or serum media. If not, the organisms should be

spread on the cover-glass mixed with a drop of serum, or better, with a drop of one of the diluted serum media. Dry in the air and fix by heat.

The capsules are stained as follows: A 5 per cent. or 10 per cent. aqueous solution of gentian violet or fuchsin (5 c.c. saturated alcoholic solution gentian violet to 95 c.c. distilled water) is used. This is placed on the dried and fixed cover-glass preparation and gently heated for a few seconds until steam arises. The dye is washed off with a 20 per cent. solution of copper sulphate (crystals). The preparation is then placed between filter paper and thoroughly dried (Plate III, Figs. 14 and 16).

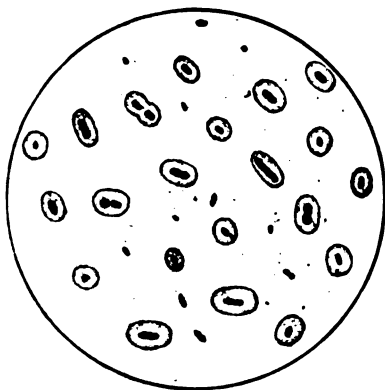


FIG. 13.—Capsule stain by Hiss's method. *Rhinoscleroma bacillus*.  $\times 1000$ . (Thro.)

**HUNTOON'S METHOD<sup>1</sup>** (Fig. 1, p. 33).—A 3 per cent. solution of nutrose is cooked for 1 hour in an Arnold and tubed unfiltered after adding 0.5 per cent. carbolic. The organisms to be stained are mixed with a drop of nutrose, spread in thin film on glass slide and dried in air, not fixed. The stain is made up of 0.5 per cent. concentrated nitric acid, 1 per cent. of a 1 per cent. acetic acid solution, 1 per cent. of alcoholic solution of basic fuchsin (or other stain), and 1 per cent. of carbol-fuchsin all in distilled water. The stain is kept on the film for 30 seconds, washed in water, and dried.

**Staining Spores and Acid-fast Bacteria.**—We have already noted that during certain stages in the growth of a number of bacteria, spores are formed which refuse to take up color when the bacteria are stained in the ordinary manner. Special methods have been devised for causing the color to penetrate through the resistant spore membrane.

In the simplest method a cover-slip after having been prepared in the usual way is covered with Ziehl's carbol-fuchsin solution and held over the Bunsen flame until the fluid steams. This is continued for one or two minutes. It is then washed and dipped in a decolorizing acid solution, such as a 2 per cent. alcoholic solution of nitric acid, or a 1 per cent. solution of sulphuric acid in water, until all visible color has disappeared, then it is washed and dipped for one-half minute in a saturated watery solution of methylene blue. The bodies of the bacilli are blue and the spores red. This same method is also used for staining acid-fast bacilli. Sometimes the spores refuse to take the stain in this manner. We then can adopt *Moeller's method*, which is designed still further to favor the penetration of the coloring matter through the spore membrane. The prepared cover-slip is held for two minutes in chloroform, then washed off in water, and placed from one-half to three minutes in a 5 per cent. solution of chromic acid, again washed off in water, and now stained by car-

<sup>1</sup> Personal communication

bol-fuchsin, which is steamed for several minutes. The staining fluid is then washed off and the preparation decolorized in a 3 per cent. solution of hydrochloric acid or a 5 per cent. solution of sulphuric acid. The preparation is finally stained for a minute in methylene-blue solution. The spores will be red and the body of the cells blue. The different spores vary greatly in the readiness with which they take up the dyes, and we have, therefore, to experiment with each variety as to the length of time it should be exposed to the maceration of the chromic acid. Even under the best conditions it is almost impossible to stain some spores.

*Spore Stain.*—Huntoon has reported the following rapid and reliable method:

4 gm. acid fuchsin (Grübler) dissolved in 50 c.c. 2 per cent. aqueous acetic acid.

2 gm. methylene blue (Grübler) dissolved in 50 c.c. 2 per cent. aqueous acetic acid.

Mix the two solutions, shake and set aside for fifteen minutes. Heavy precipitate results. Filter mixture through well-moistened filter paper. Use the filtrate for staining. Reddish-purple filtrate will keep for several weeks. Refilter if precipitate appears.

*To Stain.*—Make a rather thick smear, preferably from an agar slant. Cover smear with dye and steam one minute. Wash in water. Film appears bright red. Dip slide in dilute solution of sodium carbonate (7 or 8 drops saturated solution in tumblerful of water). When film turns blue, rinse immediately in water. Dry and examine. Spores are stained red, the body of the bacillus is stained blue. Plate III, Fig. 22, shows stained spores.

**THE HERMANN STAIN FOR ACID-FAST BACILLI.**—A, crystal violet 3 per cent. solution in alcohol; B, ammonium carbonate 1 per cent. solution in water; mix 1 part of A with 3 parts of B just before using; steam three minutes, decolorize with 10 per cent. nitric acid, wash in alcohol, and counter-stain in Bismarck brown. (See Plate VI.)

**Staining Flagella.**—For the demonstration of flagella, which are possessed by all motile bacteria, we are indebted first to Löffler. The staining of bacterial flagella well is one of the most difficult of bacteriological procedures. In all methods young (twelve-to-eighteen-hour) cultures of agar should be chosen. Enough of the culture to produce slight cloudiness is placed in a few cubic centimeters of filtered tap water in a test-tube. This may be used immediately, or allowed to stand in the thermostat at blood heat for from one to two hours to permit slight development. A tiny drop of this rather thin emulsion is allowed to spread with as little manipulation as possible over the cover-glass so that it may dry quickly. This latter point seems to be the important one, since slow drying allows the bacteria to shed their flagella.

*Bunge's modification of Löffler's method* is carried out as follows: Cover-glasses which have been most carefully cleaned are covered by a very thin smear. After drying in the air and passing three times through the flame, the smear is treated with a mordant solution, which is prepared as follows: To 3 parts of saturated watery solution of tannin add 1 part of a 25 per cent. solution of ferric chloride. This mordant should be allowed to stand for several weeks before using. After preparing the cover-slip with all precautions necessary to cleanliness, the filtered mordant is allowed to act cold for five minutes, after which it is warmed and then in one minute washed off. After drying, the smear is stained with the carbol-fuchsin or carbol-gentian violet solution, and then washed, dried, and mounted. (Plate III, Figs. 18-21.)

Frequently the flagella appear well stained, but often the process has to be

repeated a number of times. Overheating of the film prevents the staining of the flagella. The cell membrane may also show by this method.

*Van Ermengen's method* gives good results. It is as follows: The films are placed for one hour at room temperature, or are heated for five minutes over a water-bath at 100° C. in the following solution:

#### SOLUTION A.

Osmic acid, 2 per cent. solution . . . . .	1 part.
Tannin, 10 to 25 per cent. solution . . . . .	2 parts.

Wash successively with water, absolute alcohol, and water, then place in the following solution for a few seconds:

#### SOLUTION B.

0.5 per cent. solution of AgNO<sub>3</sub> in distilled water.

Without washing transfer them to a third solution:

#### SOLUTION C.

Gallic acid . . . . .	5 grams
Tannin . . . . .	3 grams
Fused potassium acetate . . . . .	10 grams
Distilled water . . . . .	350 c.c.

After keeping in this for a few seconds, place again in Solution B until film begins to turn black. Then wash and examine.

**Eosin-methylene-blue Stains.**—These are polychrome staining mixtures introduced by Nocht, Romanowsky and others chiefly for the staining of animal cells, but they are also useful in differentiating bacteria and other germs in tissues and exudates, especially in differentiating those organisms that take ordinary stains faintly, such as the spirochetes. They are fine differential stains for chromatin. Many modifications have been proposed.

In a study of the essential constituents of the Romanowsky stain MacNeal says, both methylene azure and methylene violet are present and participate in the nuclear staining. The preparation of solutions directly from the pure dyes, methylene azur, methylene violet, methylene blue and eosin, has been recommended as the best manner of preparing these staining solutions as the proportion of the various constituents may be varied at will to obtain various kinds of differentiation. As a routine blood stain for study of leukocytes and staining of hematozoa, the following is recommended by MacNeal:

#### SOLUTION A.

Methylene azur . . . . .	0.3
Methylene violet (Bernthsen's, insoluble in water) . . . . .	0.1
Methylene blue . . . . .	2.4
Methyl alcohol, pure . . . . .	500.0

#### SOLUTION B.

Eosin, yellowish, water soluble . . . . .	2.5
Methyl alcohol, pure . . . . .	500.0

These solutions keep for at least a year. They are mixed in equal parts and diluted by the addition of 25 c.c. of methyl alcohol to each 100 c.c. of the mixture. This final mixture is employed in the same manner as Leishman's stain. It keeps for a few months.

*Giemsa's Method.*—Smears are fixed in neutralized methyl alcohol for one minute. There are several variations of Giemsa's method. Two of them are given here:

## I.

Azur II—Eosin . . . . .	3.0 grams
Azur II . . . . .	0.8 gram.
Glycerin (Merck, chem. pure) . . . . .	250.0 c.c.
Methyl alcohol (chem. pure) . . . . .	250.0 c.c.

Both glycerin and alcohol are heated to 60° C. The dyes are put into the alcohol and the glycerin is added slowly, stirring. The mixture is allowed to stand at room temperature overnight, and after filtration is ready for use.

The solution is prepared ready for use by Grübler, Leipzig.

One drop of the stain to every cubic centimeter of distilled water, made alkaline by the previous addition of 1 drop of a 1 per cent. solution of potassium carbonate to 10 c.c. of the water, is poured over the slide and allowed to stand for one-half to three hours. The longer time brings out the structure better, and in twenty-four hours well-made smears are not overstained. After the stain is poured off, the smear is washed in running tap-water for one to three minutes, and dried with filter paper. If the smear is thick, the organisms may come out a little more clearly by dipping in 50 per cent. methyl alcohol before washing in water; then the washing need not be so thorough. By this method of staining, the cytoplasm of protozoa stains blue and the nuclear substance a blue red or azur. Young bacteria usually take a dark purple stain, and their metachromatic granules an azur.

## II.

- 1.0 gm. azur I (Grübler) in 1000 c.c. distilled water.
- 0.8 gm. azur II (Grübler) in 1000 c.c. distilled water.
- 1.0 gm. French eosin in 100 c.c. distilled water.

To mix for use.

- 9.0 c.c. azur I solution.
- 9.0 c.c. azur II solution.
- 0.15 c.c. French eosin solution.
- 40.0 c.c. distilled water.

Stain one and a half hours at incubator temperature.

*Wright's Stain.*—One per cent. methylene blue (alcohol rectified), and 0.5 per cent. sodium carbonate are mixed and placed in a steam sterilizer for one hour. When cold, 0.1 per cent. solution of extra B-A eosin (500 c.c. eosin to 600 c.c. methylene-blue solution), is added until the mixture becomes purplish, and a finely granular black precipitate appears. This precipitate is filtered off and dried without being washed. A saturated solution of this is made in pure methyl alcohol. This is filtered and then diluted by adding to 40 c.c. of it 10 c.c. of methyl alcohol. In using, a few drops are placed on the film for a minute; then water is dropped on until a greenish iridescence appears. The stain then remains on for two minutes; then is washed off with distilled water, allowing a little to remain on until differentiation is complete. Dried with filter paper.

*Leishman's Stain.*—This is a modification of *Jenner's stain* which is simply a solution of eosin and methylene blue in methyl alcohol. Instead of ordinary methylene blue, Leishman used the active constituents formed in this stain.

*Solution A.*—To a 1 per cent. solution of medicinally pure methylene blue in distilled water add 0.5 per cent. sodium carbonate and heat at 65° C. for twelve hours, then allow it to stand ten days at room temperature.

*Solution B.*—Eosin extra B-A (Grübler) 0.1 per cent. solution in distilled water.

Mix Solution A and B in equal amounts and allow to stand six to twelve hours, stirring at intervals. Filter and wash the precipitate thoroughly. Collect, dry and powder it. 0.15 gram is dissolved in 100 c.c. of pure methyl alcohol to form the staining solution. It keeps perfectly for at least five

months. To stain, cover the dried but unfixed film of blood with the staining solution. After thirty to sixty seconds add about an equal amount of distilled water. Allow this mixture to act for five minutes. Wash in distilled water for about one minute, examining the specimen mounted in water under the microscope. Blot, dry thoroughly, mount in balsam, or preserve the specimen as an unmounted film.

**Methods of Staining Spirochetes.**—Giemsa's method gives excellent results. *Goldhorn's Method*, which also gives fine results, is a modification of Giemsa's. It is as follows: Dye; water, 200 cm.; lithium carbonate, 2 grams; methylene blue (Merck's medicinal or a similar preparation), 2 grams. This mixture is heated in a rice boiler with a moderate amount of heat until a rich polychrome has formed. This is determined by examining a sample against artificial light and noting the appearance of a distinctly red color. The solution is allowed to cool and the residue is removed by filtering through cotton. To one-half of this filtrate 5 per cent. acetic acid is gradually added until a strip of litmus paper shows above the line of discoloration a distinct acid reaction. The remaining half of the dye is now added, so as to carry the reaction back to a low degree of alkalinity. A 0.5 per cent. French eosin solution is now added gradually, while the mixture is being stirred until a filtered sample shows a pale bluish color with slight fluorescence. The mixture is allowed to stand for one day and filtered. The precipitate is collected on a double filter paper and dried at a temperature not exceeding 40° C. It is then removed from the filter paper and dissolved in commercial wood alcohol. It is allowed to stand for one day in an open vessel and then filtered.

To use the stain on smears sufficient dye to cover the smear is dropped on an unfixed preparation and allowed to remain for three or four seconds; the excess is then poured off. The slide is now introduced slowly into clean water with the film side down, is held there for four or five seconds and is then shaken in the water to wash off the excess of dye. It is then allowed to dry and is ready for examination. The pallidum stains violet.

**Silver Impregnation Method.**—*In Smears.*—Until recently the demonstration in smears of the syphilis spirochete by the *silver impregnation method*, so successfully used by Levaditi in section, has been unsatisfactory. Stern, however, and Flexner corroborating him, have gotten beautiful results by the following simple method:

- (a) Air-dried in 37° incubator for some hours.
- (b) Ten per cent. aqueous silver nitrate for some hours (Flexner thinks three or four days' exposure better) in diffuse daylight.
- (c) When the brownish color reaches a certain tone (easily recognized after experience) and when a metallic sheen develops, the slide is washed well in water, dried, and mounted.

The blood cells are well preserved, they have a delicate dark brown contour, and contain fine light brown granules. The spirochetes are deep black on a pale brown and in places a colorless background.

Other spirochetal organisms may be silvered by this method, but as they may be differentiated with greater difficulty than with Giemsa's stain, the latter should always be used as well.

These organisms may also be demonstrated by the India-ink method (see p. 74).

The flagella are brought out by Löffler's method or by the stain recommended by Goldhorn.

**In Sections.**—Sections are prepared by the *silver impregnation method of Levaditi* (Levaditi and Manouelian, 1906) as follows: Fix small pieces of tissue 0.5 mm. in thickness for twenty-four to forty-eight hours in formalin, 10 per cent. Wash in 95 per cent. alcohol twelve to sixteen hours. Wash in distilled water until the pieces sink. Impregnate two or three hours at room temperature and four to six hours at 50° C. in the following fluid: Nitrate of silver, 1; pyridine, 10 (add just before using); distilled water, 100. Wash rapidly in 10 per cent. pyridine. Reduce the silver by placing in the following mixture for several



hours: Pyrogallic acid, 4; acetone, 10 (add just before using); pyridine, 15; distilled water, 100. Harden in alcohol; xylol; paraffin. Levaditi's first method is longer but more reliable. Fix small pieces in formalin, 10 per cent. Harden in 95 per cent. alcohol. Wash in distilled water several minutes. Impregnate three to five days at 37° C. in 1.5 per cent. solution silver nitrate. Reduce twenty-four hours in: Pyrogallic acid, 4; formalin, 5; water, 100. Imbed in paraffin. By these methods the spirochetes appear densely black.

**Ross's Method of Examining a Large Quantity of Malarial Blood in One Film.**—A large drop of blood (about 20 c.mm.) is placed on a glass slide and is slightly spread over an area which can be covered by an ordinary cover-glass. This is allowed to dry in the air or it is warmed over a flame without heating it more than enough to fix the hemoglobin. The dry film is then covered with an aqueous solution of eosin (10 per cent.) and allowed to remain about fifteen minutes. This is then gently washed off and a weak alkaline methylene-blue solution is run over the film and left for a few seconds, when the preparation is again gently washed. After drying, it is ready for examination.

**The Eosin-methylene-blue Method Recommended by Mallory for Tissues may be Used for Smears as Follows.**—The smears are fixed in Zenker's solution for one-half hour; after being rinsed in tap-water they are placed successively in 95 per cent. alcohol + iodine, one-quarter hour; 95 per cent. alcohol, one-half hour; absolute alcohol, one-half hour; eosin solution, twenty minutes, rinsed in tap-water; methylene-blue solution, fifteen minutes, differentiated in 95 per cent. alcohol from one to five minutes; and dried with filter paper.

**Staining Method for Negri Bodies (Williams's Modification of Van Gieson's Method).**—Smears partially air-dried are fixed for ten seconds in neutral methyl alcohol to which 0.1 per cent. picric acid has been added. Excess of fixative removed by filter paper. Smears then stained in following solution: Saturated alcoholic solution fuchsin, 0.5 c.c.; saturated alcoholic solution methylene blue, 10 c.c.; distilled water, 30 c.c. The stain is poured on the smear and held over the flame until it steams. The smear is then washed in tap-water and blotted with fine filter paper. The Negri bodies are magenta, the nerve cells blue, and the red blood cells yellow, or salmon color (Plate X, Fig. 1). This staining mixture may be kept in the ice-box for a long time.

**Heidenhain's Iron-hematoxylin Stain.**—(a) Mordant and differentiating fluid: Iron oxyammonium sulphate, 2.5 grams; distilled water, 100 c.c. (b) Staining fluid: Hematoxylin, 1 gram; alcohol, 10 c.c.; distilled water, 90 c.c. (To be kept in a red bottle and allowed to stand for about four weeks before using.) For use see chapter on Amebæ.

**Preservation of Smears.**—Dry stained preparations of bacteria keep indefinitely, but if mounted in Canada balsam, cedar oil, or dammar lac they tend gradually to fade, although many preparations may be preserved for many months or years. Dry unstained spreads should be kept in the ice-box until stained.

**Examination of Microörganisms in Tissues.**—Occasionally it is of importance to examine the organisms as they occur in the tissues themselves. The tissues should be obtained soon after death, so as to prevent as much as possible postmortem changes, with consequent increase or decrease in the number of microbes. Selected pieces of tissues can be frozen by ether or carbon dioxide and sections cut, but the best results are obtained when the material is imbedded in paraffin or in celloidin.

**Fixing and Hardening Tissues.**—From properly selected portions small pieces, not larger than  $\frac{1}{4}$  inch by  $\frac{1}{4}$  inch, are removed and placed in one of the following fixatives:

1. *Absolute Alcohol.*—Absolute alcohol for from four to eight hours, and longer if thicker. For the larger pieces it is better to change the alcohol after eight hours. The pieces of tissue should be kept from falling to the bottom, as

the higher layers of alcohol remain nearer absolute. If along with the micro-organisms one wishes to study the finer structure of the tissue, it is better to employ one of the other fixatives.

2. *Formalin*.—For fixing in formalin the tissue is put in 4 to 10 per cent. formalin solution for three to twenty-four hours, and then in successive strengths of alcohol.

3. *Corrosive Sublimate*.—Corrosive sublimate (saturated solution in 0.75 per cent. sodium chloride solution) is an excellent fixing agent. Dissolve the sublimate in the salt solution by heat, allow it to cool; the separation of crystals will show that saturation is complete. For pieces of tissue  $\frac{1}{4}$  inch in thickness four hours' immersion is sufficient; for larger, twenty-four hours may be necessary. They should then be placed in pieces of gauze and left in running water for from twelve to twenty-four hours, according to the size of the pieces, to wash out the excess of sublimate.

4. *Sublimate Alcohol*.—Hot sublimate (saturated) alcohol (50° C.) or saturated sublimate, to which 5 per cent. glacial acetic acid may be added. The preparation should remain in it a few seconds, then should be washed for one-half hour in 60 per cent. iodine-alcohol, and then placed in 70 per cent. alcohol. They may remain here for an indefinite time, until they are to be stained, when they are rinsed in distilled water and then placed in the staining fluid.

5. *Osmic Acid*.—Two per cent. osmic acid (to be kept in a red glass with a ground-glass stopper). Moist smears are exposed to its fumes for a few seconds, small pieces for sections, four to eight hours, then carried through the various alcohols and xylol and mounted or imbedded in the usual way.

6. *Hermann's Fluid*.—A 1 per cent. solution platinum chloride, 15 c.c.; a 2 per cent. solution osmic acid, 4 c.c.; glacial acetic acid, 1 c.c. Moist spreads may be fixed for several minutes; very small pieces of tissue for twenty-four hours.

7. *Zenker's Fluid*.—Add to a solution of Müller (potassium bichromate, 2 to 2½ parts; sodium sulphate, 1 part; water, 100 parts) 5 per cent. of saturated sublimate solution and, when ready to use, 5 per cent. of glacial acetic acid. Moist spreads are fixed for one to five minutes, small pieces of tissue for three to twelve hours. They are then washed with water or put immediately into successive alcohols.

To *harden* they are placed successively for twenty-four hours each in the following strengths of ethyl alcohol: 30 per cent., 69 per cent., and 90 per cent. Finally they are placed in absolute alcohol for twenty-four hours which dehydrates them and they are then ready to be imbedded in paraffin.

To *imbed in paraffin*, the pieces are put in (1) cedar oil until translucent; (2) cedar oil and paraffin, equal parts, at 37° C. for two hours; (3) paraffin 52° C. two hours in each of two baths. They are then boxed ready for sections. Sections are cut at 3 to 6 $\mu$ , and are dried at 36° C. for about twenty-four hours, protected from dust. Xylol may be used instead of cedar oil.

The paraffin sections of tissue having been prepared and cut, they are ready for staining after the paraffin is removed. If all of the sublimate has not been removed by the water, the sections may be immersed in iodine-alcohol for ten minutes.

**LÖFFLER'S STAINING METHOD FOR SECTIONS.**—The section is placed in Löffler's alkaline methylene-blue solution for five to thirty minutes, decolorized for a few seconds in 1 per cent. acetic acid. It is then placed in absolute alcohol, xylol, and Canada balsam. The number of seconds during which the preparation remains in the acetic acid must be tested by trials.

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## CHAPTER IV.

### GENERAL METHODS USED IN THE CULTIVATION OF MICROÖRGANISMS.

#### CULTURE MEDIA, METHODS OF ISOLATION AND CULTIVATION, CULTURAL CHARACTERISTICS.

THE methods employed for the artificial cultivation of microörganisms are of fundamental importance. By their use we can obtain one variety growing apart from all others, namely, in pure culture. This pure culture may be planted on various media and the morphological, biochemical, and cultural characteristics studied for classification and identification. It is evident that all glassware and instruments used must be free from other microörganisms; that is, they must be sterile.

**Preparation of Glassware.**—Various types of glassware, such as test-tubes, flasks, bottles, beakers, pipettes, etc., are used (see Figs. 14 to 22). New glassware, as a rule, only requires mechanical cleansing with soap and water, loosening adherent dirt with test-tube or bottle brushes. Old glassware containing cultures should be sterilized either in the autoclave or in a covered boiler. In the latter case the tubes are covered with water, about 5 per cent. of washing soda or soap added, and boiled for one hour. Any solid medium present is melted and the glassware can then be washed according to the directions already given. After washing, drain and allow to dry.

**Neutralization of Glassware.**—Where slight changes of reaction are important the glassware should be neutralized after washing. Soak in 1 per cent. hydrochloric acid for several hours or boil for one-half hour, wash free of acid, and rinse in distilled water. New glassware, especially the cheaper grades, is most likely to give off free alkali.

The clean glassware is plugged loosely with ordinary non-absorbent cotton. The cotton should not be twisted in, as creases will form along the glass, leaving channels for contamination. Either fold into a plug or take a square of cotton, fold one corner, place a rod against the fold, and push the cotton into the neck of the container or tube. A sufficient amount of cotton should project for handling and the plug should be just tight enough to allow one to lift the container by means of the plug. Several thicknesses of filter paper may be used to cover beakers and other wide-mouthed containers.

**Sterilization of Glassware.**—All glassware is sterilized by dry heat. This is done after plugging. Some type of hot-air sterilizer is used (Fig. 23). Heat to 160° C. for one hour. Leave the sterilizer closed when the sterilization is finished, so that cooling is gradual or the glassware may crack. This heating not only sterilizes but sets the shape of the cotton plugs.

#### CULTURE MEDIA.

**General Considerations.**—Most microörganisms causing disease require complex foodstuffs similar in constitution to those in the animal body. The general basis of media for these types is an extract or infusion of meat. To this may be added peptone and sodium

chloride. Some may require uncoagulated proteins, such as serum or blood, or even fresh tissues. Carbohydrates may be added. The non-pathogenic microörganisms vary in their ability to grow on these more complex media, and some will only grow on simple media containing



FIG. 14.—Test-tubes can be also used without lip. Average size, 6 x  $\frac{1}{4}$  inches.



FIG. 15.—Beakers.

inorganic salts. Media may be either fluid or solid. In the latter case there is added some jelly substance such as gelatin or agar or an albumin which is coagulated by heating.



FIG. 16.—Globe flask.

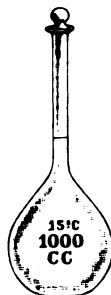


FIG. 17.—Volumetric flask.



FIG. 18.—Erlenmeyer flask.

Certain technical methods are employed in the preparation of nearly all culture media, such as adjustment of reaction, clearing, filtering, etc. These details must be understood before the actual preparation of media is given.

**Reaction of Media.—Titration and Adjustment.**—A moderately alkaline reaction to *litmus* is satisfactory for the growth of most pathogenic microorganisms. For ordinary work the usual acid reaction of the media in preparation may be reduced by the addition of a 4 per cent. sodium hydrate solution until red litmus paper is turned slightly but distinctly blue and blue litmus paper remains the same color. Litmus, however, is not a delicate indicator, and



FIG. 19.—Type of bottle commonly used for dilutions, etc., or as substitutes for flasks.



FIG. 20.—Blake bottle. Laid on its flat side it gives a large surface of broth or agar.



FIG. 21.—Petri dish. Commonest size is 10 c.c. in diameter.

*phenolphthalein* is generally used, giving us more accurate knowledge of the reaction. The neutral points of the two indicators are different, so that media which are alkaline to litmus are still acid to phenolphthalein. The materials necessary for titration and adjustments of the reaction to phenolphthalein are normal and twentieth normal solutions of sodium hydrate and of hydrochloric acid, a 0.5 per cent. solution of phenolphthalein in 50 per cent. alcohol, burettes, casserole, and stirring rod.

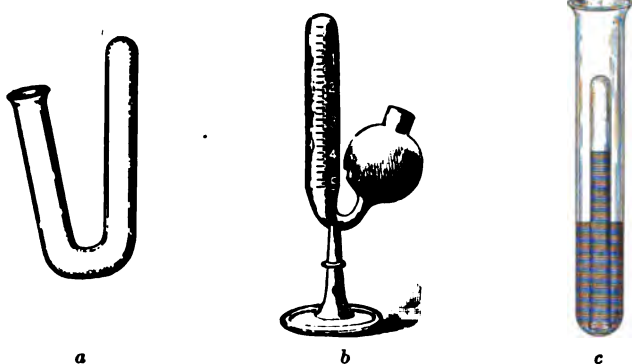


FIG. 22.—Types of fermentation tubes.

**Methods of Titration.**—Two methods may be used: (a) *room temperature* and (b) *boiling temperature*. The former is the more accurate if the medium to be tested has previously been heated to the boiling-point during its preparation. Under these circumstances with this method the reaction of a medium is set at a temperature more nearly approximating that at which the substance will be used, 37° C. of the incubator. It is *essential*, however, to use method b

*boiling temperature*, when titrating meat juice which has not been heated above  $50^{\circ}\text{C}$ . (or up to the boiling-point) for the purpose of dissolving added peptone and salt. The boiling in the casserole is needed to approximate the later conditions when the medium is boiled and sterilized. This method of titration as given below is the *standard method*. It is often misapplied, however, when used for the titration of media already heated to the boiling-point. Another error in its results is due to the fact that the more phenolphthalein is heated, the less sensitive and accurate it is as an indicator.

*Technic.—Room temperature method:* 5 c.c. of medium to be tested, 45 c.c. of distilled water<sup>1</sup> and 1 c.c. of the phenolphthalein solution are mixed in a casserole. (When agar is titrated, the water should be warmed to about  $30^{\circ}\text{C}$ . before adding the hot agar.) If no pink color is present, the medium is acid.



FIG. 23.—Hot-air sterilizer. Lautenschläger form.

While the mixture sibeing stirred twentieth-normal solution of sodium hydrate ( $\frac{N}{20}\text{NaOH}$ ) from a burette is run in until a delicate pink tinge is observed. This color should not disappear with stirring. (See page 91 for the calculation for correction.)

*Boiling Temperature.—Standard method:* 5 c.c. of medium to be tested and 45 c.c. of distilled water are mixed in the casserole and boiled for one minute, then 1 c.c. of the phenolphthalein solution is added. If no color is present the medium is acid and while hot the twentieth-normal solution of sodium hydrate ( $\frac{N}{20}\text{NaOH}$  solution) is run in from a burette until a faint but distinct pink color appears. This color must remain on stirring, otherwise more alkali

<sup>1</sup> Distilled water, freshly boiled and cooled, should be used for very accurate results, since the  $\text{CO}_2$  has been driven off by the boiling and is not reabsorbed within a few hours.

is needed. From the amount added we determine the acidity of the medium and estimate how much normal ( $\frac{N}{20}$ ) solution of NaOH must be added to obtain the reaction desired, for example:

Five c.c. required 2.4 c.c. of  $\frac{N}{20}$  NaOH to neutralize, therefore 100 c.c. (twenty times as much) would require 2.4 c.c. of  $\frac{N}{20}$  NaOH (twenty times as strong), in other words, the medium is 2.4 per cent. acid to phenolphthalein (+2.4 per cent.). Assuming we desire a reaction of +1 per cent., we must then add 2.4 c.c. — 1 c.c. or 1.4 c.c. of  $\frac{N}{20}$  NaOH to every 100 c.c. of medium or 14 c.c. to a liter.

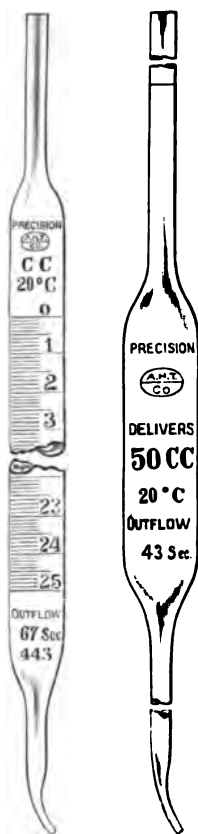


FIG. 24  
Graduated Volumetric  
pipette. pipette.  
Obtainable in various sizes and graduations

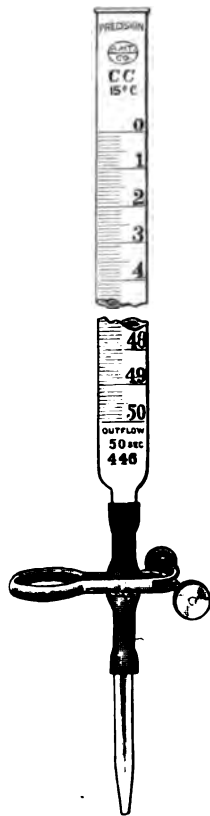


FIG. 25  
Burettes. Most convenient type has a blue line against a white background on back, giving sharper readings.

Should, on the other hand, the mixture in the casserole show a pink color then the medium is alkaline and  $\frac{N}{20}$  HCl is used, heating as above, until only a faint pink color persists. If we use 0.5 c.c., then the medium is 0.5 per cent. alkaline (—0.5 per cent.), and it will require 0.5 c.c. of  $\frac{N}{20}$  HCl for every 100 c.c., or 5 c.c. for every liter to bring it to the neutral point. But we want the reaction to be +1 per cent. or 1 per cent. acid, we therefore add 5 c.c. plus 10 c.c. or 15 c.c. of  $\frac{N}{20}$  HCl to each liter.

After the first correction of the reaction by the addition of normal alkali or acid the medium should be again titrated after further boiling or treatment in



the autoclave. Additional correction will usually be necessary, for each time meat infusion is boiled, or heated under pressure, the reaction rises somewhat in acidity. This may have to be repeated several times before the reaction desired is obtained. Various combinations, precipitation, etc., occur which make this necessary. After experience a suitable excess of normal soda solution may be added to allow for the rise in acidity which will occur by the time the final sterilization is ended. This rise is not so marked in media prepared with beef extract (Liebig's) as a basis.

The reaction of media should always be adjusted before filtration, and after adjustment the medium must be heated for three to five minutes or precipitation will occur on subsequent sterilization, especially if sterilized in the autoclave, due to the fact that the temperature is higher than that employed during the adjustment of the reaction.

**Clearing Media.**—This is done by coagulation of an albumin, which, as it coagulates, enmeshes the fine particles. In certain methods the coagulation of the soluble albumins in the watery extract of the meat clears the medium when heated. Under other conditions an albumin such as egg is added. If the medium is hot, it must first be cooled to below 60° C. One or two eggs are used for each liter. They are broken in a small pan and mixed with a small quantity of water by means of an egg-beater. This is then added to the medium, stirring thoroughly. The medium is then heated usually in the Arnold sterilizer or autoclave, to coagulate the egg albumens.

**Filtering Media.**—For fluid media, paper or cotton is used. For media which solidify on cooling, cotton is preferable. Where media are cleared, the coagulated albumins which settle on the paper or cotton act as a filtering medium. If paper is used, it should be folded or a corrugated funnel employed. If cotton is used, a spiral of wire or a perforated porcelain plate is placed in the funnel to support the cotton and two strips of cotton laid crosswise in the funnel, the torn ends extending up the side of the funnel. Before filtration



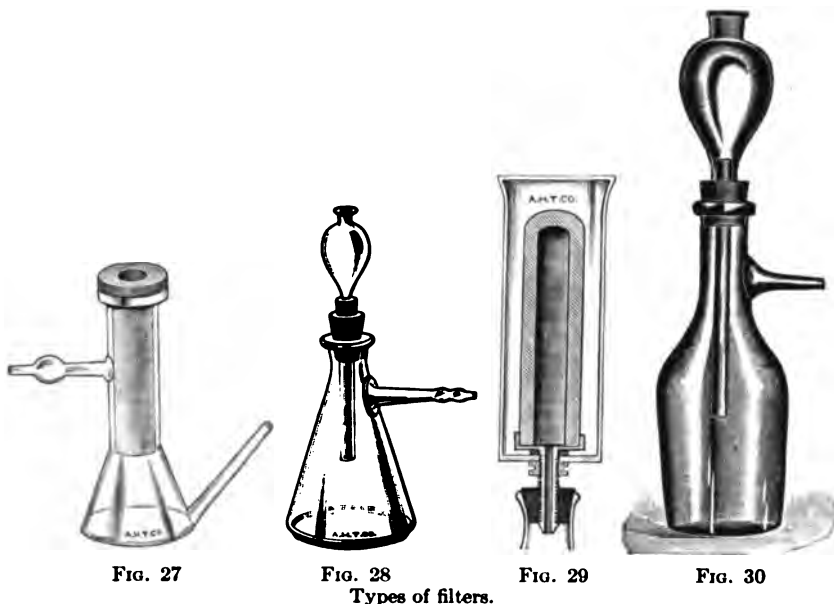
FIG. 26.—Hot-water funnel:  
a, point heat; b, inlet for water.

the paper or cotton is wet with water so that any fat in the medium will not pass through. In wetting the cotton, moisten the torn ends so they adhere to the glass and then carefully moisten the rest. The medium is poured into the funnel along a glass rod, which is helpful in holding the cotton in place when this is used. The filtrate is poured back until it comes through clear. In the case of media which solidify on cooling, the funnel should be heated before adding the medium and the medium awaiting filtration kept hot. The use of a hot-water funnel to keep the funnel and its contents hot is a great advantage. Failing this, the flask and funnel may be placed in an Arnold sterilizer.

**Preparation of Media for Use.**—After filtration the medium can be either placed in flasks for storage or placed in various containers for immediate use and sterilized. In filling, care should be taken not to wet the necks of the containers or the cotton plugs will stick. Test-tubes and other small containers are best filled from a funnel with a stop-cock or fitted with rubber tube and glass tube with pinch-cock. The amount in each container will depend on the use to which it is to be put. For the ordinary 15 cm. (6-inch) test-tube a depth of media of about 3 to 5 cm. is sufficient. If plates are to be

poured, at least 10 c.c. should be placed in a tube. If the medium is to be slanted in the tube, less is required. The slants may be made immediately after sterilization or the media melted and slanted as required. To make the slants the tubes should be laid in a row on a table with a glass rod or strip of wood to

raise the upper end. Care should be taken that the slant does not touch the cotton, and sufficient media be present in the bottom of the tube ("butt") so that on raising the tube the slant will not fall down. The medium should be well set before raising.



**Sterilization of Media.**—Sterilization may be accomplished by filtration through various types of filters which do not allow the passage of bacteria, by heat, and occasionally by chemicals, as chloroform. Sterilization by heat may be accomplished in several ways—namely, intermittent (fractional) sterilization at 100° C. or at 60° to 80° C., and sterilization by steam under pressure.

**Filtration.**—This method is resorted to where the application of heat will injure the solution, as in the case of some sugars, or where the necessary amount of heat would cause coagulation, as in the case of tissue extracts or serum. Various types of filters made of unglazed porcelain or compressed diatomaceous earth are used. Types of these filters are the Berkefeld, Pasteur, Chamberland, and Doulton filters. The bacteria are held back because of the fineness of the pores. Various grades of fineness are procurable in some brands, and on this depends the rapidity of filtration. In any case the permeability of the filter should be tested before use by filtering a broth culture of some small micro-organism, such as *Bacillus prodigiosus*. Before filtration the fluid to be filtered should be rendered as clear as possible by filtration through paper or if necessary through paper pulp. The latter is prepared by soaking torn-up



FIG. 31.—Filter pump for attachment to water faucet.

filter paper in water and then placing a layer in a porcelain filter, over a perforated porcelain plate, draining off the water and packing the layer tight. The depth of the layer will depend on the fluid to be filtered, its density, and the fineness of the particles to be removed. This should be fastened in the neck of a filtering flask and suction applied to hasten the filtration. Similar results may be obtained by filtration through sand.

The filtration through a filter candle (the size selected will depend on the amount to be filtered) is accelerated by the use of suction or pressure. Suction may be secured by the use of a filter pump attached to the hydrant or other types of suction apparatus. Pressure may be obtained by air under pressure from an installed system or by a hand pump and cylinder to equalize the pressure. A manometer is employed to determine the pressure. The accompanying cuts show the method of setting up the apparatus (Figs. 27-31).



FIG. 32



FIG. 33

FIGS. 32 and 33.—Arnold steam sterilizer. Two types.

The filter candle and all attachments with which the fluid will come in contact after passage through the candle must be sterile. The glassware can be sterilized in the hot-air oven and the filter candle and rubber connections can be sterilized by boiling for one hour or by steam under pressure. (See Autoclave.) After use the filter candle should be freed of all soluble material, especially coagulated matter, by running through it an abundance of clear water. If used for infective matter, it can then be sterilized by boiling. In any case the surface should be lightly scrubbed with a fine brush after use.

A new filter should be cleansed before use by filtering clear water. It should then be placed in cold water and boiled thoroughly.

After continued use the candles gradually become clogged. They can be renewed in some degree by careful heating to glowing in an oven. This is apt to produce fissures, hence the filter should be retested before use.

*Heat.*—*Intermittent ("Fractional") Sterilization.*—This method has two divisions:

1. That of applying heat at a temperature of 100° C. or close to it, as in the Arnold sterilizer, on three or more successive days, to media containing special

sugars, etc. The daily period of heating depends upon the size of the container; test-tubes of media require twenty minutes, whereas 1- to 2-liter flasks require forty-five to sixty minutes to allow heat to penetrate.

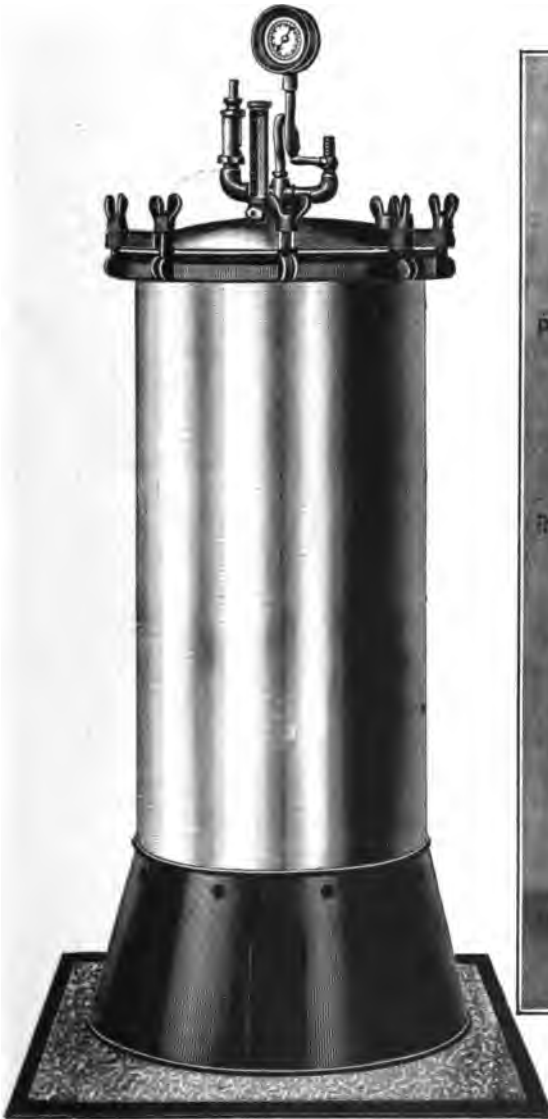


Fig. 34.—Upright type.

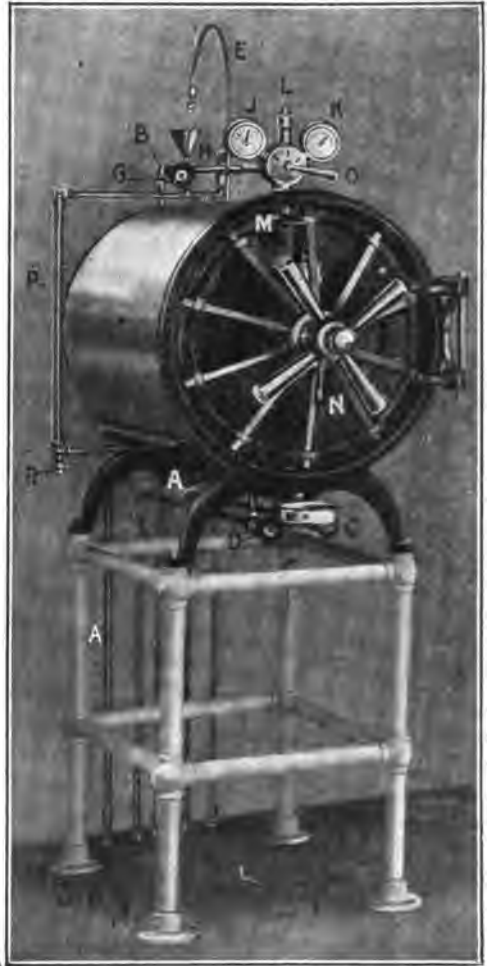


Fig. 35.—Horizontal type.

FIGS. 34 and 35.—Autoclaves may be heated by direct application of heat, or by steam under pressure, when available.

2. The application of temperatures of only  $60^{\circ}$  to  $80^{\circ}$  C. on successive days to such substances as blood serum, transudates from body cavities (*e. g.*, ascitic fluid), etc.

The principle underlying fractional or intermittent sterilization is as follows: All bacteria when free from spores are killed by exposure for one or two hours to only 60° to 80° C. heat. If the material is left overnight at about 22° C., the spores, if present, will develop to bacteria and be destroyed by the second heating. Some of the bacteria may produce spores before the second heating, or some of the spores may be slow in developing and escape. For this reason a third heating, or with the low temperatures, five or six heatings on successive days may be necessary. When this method is applied to albuminous material before coagulation, such as sera, tissue extracts, etc., the temperature selected should be below the coagulation-point of the material, usually about 60° C. All material to be treated in this manner should be as free from contamination as possible. The method has its best application to originally sterile material which, because of necessary manipulation may have become slightly contaminated. The heating may be done in a water bath or in a water-jacket oven.

**Sterilization by Steam under Pressure.**—This is done by means of an autoclave. Various forms are available which may be heated by gas or by steam (Figs. 34 and 35). All air should be displaced by the steam before closing the vent. Too rapid reduction of pressure will cause the media to boil up and wet or even blow out the plugs. The time and pressure employed will depend on conditions. As a rule 15 pounds' pressure is employed and twenty minutes are sufficient for media in test-tubes. Flasks should be heated one-half to one hour, depending on their size. Lower pressures are at times desirable where overheating is a factor, and the heating may be repeated on two successive days. The heating should be timed from the time the pressure desired is reached. The temperature will vary with the pressure thus:

5 pounds' pressure =	temperature . . . . .	108.8° C.
10   "       " =	" . . . . .	115.6° C.
15   "       " =	" . . . . .	121.3° C.

**Storage of Media.**—Media after sterilization may deteriorate for two reasons, namely, contamination (especially by molds penetrating the cotton stoppers), and by drying.

The former may be avoided by keeping the media at a low temperature, 40° to 45° F., in a dry refrigerator. Where media are used in small amounts they may be kept in flasks and tubed as needed. Evaporation of the tubed media can be lessened by dipping the plugs in paraffin or by the use of rubber caps made for this purpose. The stock flasks after removal from the sterilizer may be sealed with paraffin or sealing wax. The plug should be cut off and pushed in slightly and melted paraffin poured on it, care being taken that it does not run through the plug into the media. This can be avoided if the paraffin is nearly cold enough to set. Sealing wax may be applied in the same way or rubber caps or rubber tissue tied over the neck. If the stock flasks are capped, a small pledget of cotton moistened with bichloride of mercury solution may be placed between the plug and the cap to discourage the development of molds which may adhere during manipulation. The neck of the flask should be wiped free of the bichloride before pouring out the media. This precaution is specially useful for media stored in flasks at room temperature. The necks of all containers may be protected from dust by covering with paper before sterilization or inverting tumblers over the necks of flasks.

## COMPOSITION OF CULTURE MEDIA.

**Meat Infusion.**—One pound of finely chopped meat usually beef or veal, is macerated with 1000 c.c. of water and placed in the ice-chest for eighteen to twenty-four hours, or it may be extracted by heating to a temperature not exceeding 50° C. for one hour. The infusion is then strained through cheese-cloth and the meat juice squeezed out by twisting the cloth or by means of a meat press. The fluid contains the soluble albumins, extractives, salts, carbo-

hydrates, and coloring matter of the meat. This forms the basis for the various media to be described. If this infusion is to be stored as such, it must be boiled to coagulate the albumins (which also clears it), filtered, placed in flasks, and sterilized. Many advise the neutralization to phenolphthalein<sup>1</sup> before heating, on the ground that less of the foodstuff is precipitated than by heating the very acid infusion.

As a substitute for the meat 2 to 5 grams of Liebig's extract of beef may be used for each liter of water. When media are made with this basis, they are spoken of as *meat-extract media* in contradistinction to *meat-infusion media*.

**Nutrient Bouillon.**—*Infusion Broth.*—Meat infusion 1000 c.c., peptone<sup>2</sup> 10 grams, and sodium chloride 5 grams. Warm the meat infusion to about 50° C., add the peptone and salt and stir until dissolved. A convenient way is to use one's hand, as in this way the fluid will not get too hot, and the lumps of wet peptone are easily crushed. The mixture is then boiled over the free flame to coagulate the albumins, preferably after neutralizing the excess of acid. Evaporation should be made good by the addition of water. Correct the reaction and boil again for five minutes, filter, and place in the appropriate containers for sterilization.

*Extract Broth.*—From 2 to 5 grams of Liebig's meat extract are dissolved in each liter of water as a substitute for the infusion. The further steps are the same.

**Gelatin Media.**—*Meat-infusion Gelatin.*—Meat infusion 1000 c.c., peptone 10 grams, sodium chloride 5 grams, and gelatin ("gold label") 100<sup>3</sup> grams. Dissolve as for nutrient broth. The acidity may then be reduced and the mixture then boiled or heated in the Arnold sterilizer for a half-hour. The reaction is now set and the media again heated for fifteen minutes and filtered. The clearing is done in this way by the albumins in the meat infusion. It should be tubed at once and sterilized in the Arnold sterilizer for twenty minutes on three successive days. Reheating and resterilization are to be avoided, as the media will not set after too much heating. If the gelatin media is made with meat infusion which has been heated, it must be cleared with eggs before filtration. In this case the ingredients are dissolved by heat and the reaction set. The mixture is then cooled below 50° C. and the eggs added; and then it is heated as above and filtered.

*Meat Extract Gelatin.*—From 2 to 5 grams of extract per liter is used in place of the infusion, finally clearing with eggs.

**Agar Media.**—*Meat Infusion Agar.*—Two methods may be employed. The simplest method is to take 1000 c.c. of infusion broth and add 1.5 per cent. of shredded agar. This is then dissolved by boiling over the free flame for a half to three-quarters of an hour, making up with water the volume lost by evaporation. A better way is to place the mixture in the autoclave and heat for one-half to one hour at 15 pounds' pressure, depending on the bulk. The reaction is then set, and the mixture cooled to below 50° C. and the eggs added. The mixture is again heated to coagulate the egg, either over the free flame or in the autoclave, and filtered.

Another method of preparing agar is to make a double strength meat infusion, in which is dissolved double the amounts of peptone and salt and the excess acidity neutralized. To an equal quantity of water add 3 per cent. of agar and dissolve. Cool the agar below 50° C. and mix the two solutions. Set the reaction. Then heat for one-half to three-quarters of an hour to coagulate the meat albumin, which also clears the medium, correct the reaction if necessary, and filter.

*Meat Extract Agar.*—Prepare as for extract broth, add agar and proceed as above, or dissolve agar in half the water, the other ingredients in the other half, (double strength); mix and proceed as above.

<sup>1</sup> See Reaction of Media.

<sup>2</sup> See also Martin's Peptone Solution, p. 103.

<sup>3</sup> Use 120 grams in warm weather,

**Peptone Solution (Dunham's).**—Water 1000 c.c., peptone 10 grams, and sodium chloride 5 grams. Dissolve by heating and filter. The reaction needs no adjustment for ordinary use.

**Nitrate Broth.**—Water 1000 c.c., peptone 1 gram, and nitrate-free potassium nitrate 0.2 gram.

**Milk.**—The milk used should be raw and as fresh as possible, preferably the best grade obtainable. Steam the milk in the Arnold sterilizer for a half-hour and place on ice for several hours or overnight to allow the cream to rise. By means of a siphon remove the milk from below the cream layer. This may be tubed or litmus solution added (*litmus milk*). If a good grade of milk is used, no change of reaction is necessary; if acid, sufficient sodium hydrate solution should be added to render it slightly alkaline to litmus; if very acid, the milk should be discarded.

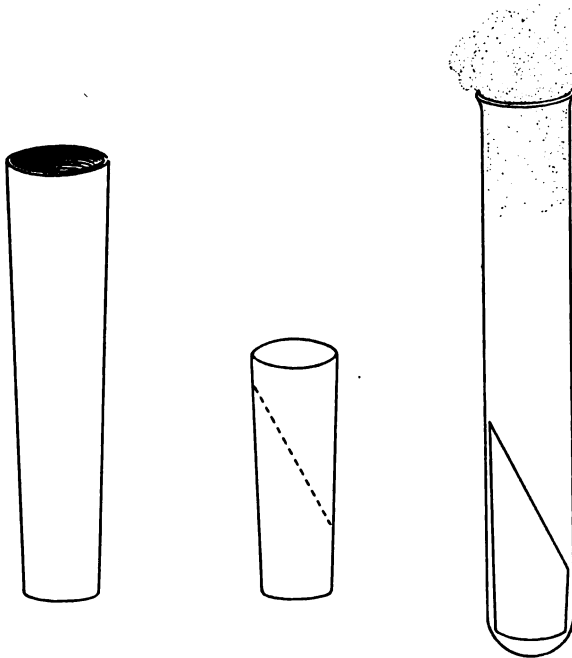


FIG. 36.—Potato borer.

FIG. 37.—Cylinder of potato, line of cut.

FIG. 38.—Finished medium.

**Potato Media.**—Large potatoes should be used and scrubbed with a nail brush under running water. Cylinders are then cut by means of an apple corer. The skin is cut off the ends of the potato cylinder and by an oblique cut, wedge-shaped pieces are obtained. A good butt should be left or the pieces will coil. While preparing the potatoes they should be kept submerged in water or the cut surfaces will discolor. As the potato is acid, the reaction must be changed. This is done by soaking in running water overnight or by soaking several hours in a 1 to 1000 solution of sodium carbonate. The pieces are then placed in test-tubes, a little water added to avoid drying, and sterilized. The test-tubes used should be 1 inch in diameter or over. If the pieces of potato are too small and thin, they will curl on sterilizing.

*Glycerin potato* for the cultivation of tubercle bacilli should be cut as above, soaked overnight in 1 to 1000 sodium carbonate solution, drained, and covered with a 5 per cent. glycerin solution for twenty-four hours. When tubed, the glycerin solution or water is added for maintaining the moisture,

**Potato Juice Media.**—*Potato Juice.*—White potatoes, grated (or run through chopping machine), 1 pound to 1 liter of water. Soak overnight. Heat to boiling. Press through cheese-cloth. Add one egg per liter. Autoclave one-half hour to clear. Filter through cotton (very tedious). Store in flasks and sterilize in autoclave one-half hour at 15 pounds' pressure.

*Agar.*—To veal agar add 5 per cent. potato juice and 5 per cent. glycerin.

*Broth.*—Meat infusion and potato juice are mixed in equal parts. Peptone and salt are added as in ordinary bouillon.

**Semisolid Media.**—Various types of semisolids are in use. The amount of agar will vary according to the use to which it is put. For slants, 0.75 per cent. to 1 per cent. of agar is necessary; for stab cultures, 0.5 per cent. or even less is sufficient. If the agar is to be diluted by the addition of serum or other enriching substance, this must be allowed for in preparing the medium. The following is an example (North): Meat infusion (1 pound of meat to 500 c.c. of water) is warmed and 20 grams of gelatin and 20 grams of peptone dissolved in it. In 500 c.c. of water dissolve 10 grams of agar and cool to below 50° C. Mix the two, adjust the reaction, and heat to coagulate the albumin and clear the medium. Readjust the reaction and reheat if necessary; filter. A very satisfactory medium can be made by simply using 0.5 per cent. of agar instead of the usual 1.5 per cent. employed. This can be diluted by the addition of one-third of its bulk of an enrichment fluid and will still set sufficiently for stab cultures. Nutrose (1 per cent.) may also be used.

**Carbohydrates in Media.**—Carbohydrates are added to media to determine whether acid or gas or both are produced or for utilization as a foodstuff to cause a more abundant growth on the part of the inoculated organisms. Only the purest carbohydrates obtained should be used. The following is a partial list of substances which may be used:

*Mono-hexoses*—dextrose, levulose, galactose, and mannose.

*Bi-hexoses*—saccharose, maltose, and lactose.

*Tri-hexoses*—raffinose.

*Polyhydric-alcohols*—erythrite (tetra), adonite (penta), and mannite, sorbite, dulcitol, and glycerin (hexa).

*Pentoses*—arabinose, xylose, and rhamnose.

*Polysaccharids*—glycogen, dextrin, and inulin.

Carbohydrates are usually added to media in 1 per cent. amounts, with the exception that 5 per cent. of glycerin is used. Besides the true carbohydrates, various glucosides, such as salicin, coniferin, etc., are used, as well as carbohydrate-like substances, like inositol. They may be used in any medium, either solid or fluid. Where meat extract or meat infusion is used as a basis, it must be remembered that this contains fermentable sugar in muscle. If the media are to be used to determine whether sugar is acted upon or not, the muscle sugar must be removed before preparing the media. The meat extract or infusion to be used should be neutralized to phenolphthalein, and for each liter add a broth culture of *B. coli* or one of its allies. This is incubated for forty-eight hours. The bacillus ferments the sugar present. The infusion is now sterilized and used in the preparation of media. Media made in this way are called "*sugar-free media*." To the sugar-free media the sugars may then be added. Serum water (Hiss) (see Serum Media) may also be used as a basis for carbohydrate media.

Many of the carbohydrates are very susceptible to heat. They are split into simpler compounds which may be fermented by an organism unable to ferment the unaltered sugar and thus lead to error. The usual method is to add sufficient sugar to sterile water to make a 10 per cent. or 20 per cent. solution and heat this in small containers in the Arnold sterilizer. This solution is then added to the media in sufficient amounts to give a final 1 per cent. content.

Certain sugars, especially some samples of maltose, are so easily split by heat that they must be sterilized by filtration.

In routine work with glucose, lactose, saccharose, mannite, and dulcitol



is usually sufficient to add the sugar to the media and sterilize by intermittent sterilization. Although there may be a slight destruction of the glucose, it is not enough to be important. Inulin is an exception. Because of the resistant spores commonly present, the inulin solution should be sterilized in the autoclave.

Fluid-sugar media, with the exception of serum-water media, are usually filled into fermentation tubes, where gas production is to be determined. Three types are used (Fig. 22, *a*, *b*, *c*, p. 89). The sterilization of these types is usually done in the Arnold sterilizer. To fill type *c* the small tube is first filled and with as little inversion as possible slipped, mouth down, into the large tube. Sufficient media is then added to allow the small tube to fill after expulsion of the air contained and leave the proper amount of media in the outside tube. The air in the inner tube is driven out during sterilization. An indicator may be added to show the changes in reaction. If litmus is used, the media should be very slightly alkaline to this indicator, or slight acid production will not become evident. In careful work, titration is the best method of determining the changes in reaction.

**Litmus and other Indicators.**—The ordinary litmus of commerce is not a very delicate indicator because of the impurities present which give it a reddish color. A purified litmus (Merck or Kahlbaum), or a solution such as that known as "Kubel and Thiemann," should be used. The purified litmus comes in the dry form and should be pulverized and added to distilled water in 5 per cent. amounts. This is steamed in the Arnold sterilizer for two hours, shaking the mixture every twenty minutes. The solution is then filtered and the filtrate sterilized. The solution must be kept sterile. Usually 5 per cent. of the litmus solution is added to the media. This may be varied to suit personal preferences. Media to which litmus is added should be carefully adjusted to a slightly alkaline reaction to litmus or slight changes will be obscured. (In place of litmus a 1 per cent. solution of Kahlbaum's azolitmin may be employed.) Litmus media may be decolorized by the growth of bacteria which rob it of its oxygen and reduce it to the colorless leukobase. The color will return on exposure to the air. Where stab cultures are used, it may be necessary to melt the medium and pour it into Petri dishes to get sufficient air exposure to cause the color to reappear. Where the color change of individual colonies is to be observed, the diffusion of any acid produced may be prevented by using 3 per cent. agar instead of 1.5 per cent.

Various aniline dyes which are reduced to their colorless leukobase by the action of sodium sulphite (Endo) or acid dyes decolorized by sodium hydroxides (Andrade) may be used as indicators. When the sugar is split by the bacteria, aldehydes and organic acids are produced, which cause the color to return. The former act most vigorously on the Endo indicator. (See Typhoid Media.) Media to be used with the Endo indicator should be alkaline to litmus. The Andrade indicator consists of 100 c.c. of a 0.5 per cent. watery solution of acid fuchsin decolorized by the addition of 16 c.c.  $\frac{N}{2}$  NaOH solution. One per cent. is added to the media. The reaction of the medium is adjusted to the indicator so that it is distinctly pink when hot but colorless when cold.

Powdered insoluble carbonates may be used in solid plating media. If the powder is evenly distributed throughout the media, acid-producing colonies will be surrounded by a clear area.

Phenolphthalein is also employed. The medium should be faintly alkaline and sufficient 1 per cent. alcoholic solution added to give a distinct pink color. The color is discharged when acid is produced.

Media containing indicators are preferably sterilized in the Arnold sterilizer. The autoclave may be used in emergencies, but the indicator is somewhat injured.

**Use of Insoluble Carbonates.**—Either crushed marble or calcium carbonate powder may be used. About 1 per cent. by bulk is sufficient. Their main application is in fluid media, especially as carbonate broth for the streptococcus-pneumococcus group.

The value of the carbonates is that they neutralize the acids that may be produced by the growth of the bacteria, and which might prevent further development or even injure the viability of the culture. Sugars may be used, therefore, in media containing carbonates, and the growth and viability of the culture thus increased. The exact manner in which the calcium helps is not known. Where the medium is used in large amounts, the containers should be shaken from time to time. Powdered calcium carbonate may also be used in plating media to limit acid diffusion.

**Media Solidified by Coagulation of Albumin.**—*Löffler's Blood Serum.*—Mix 3 parts of calf or sheep serum and 1 part of nutrient broth (neutral to litmus) to which 1 per cent. of dextrose has been added. The mixture is run into tubes and the tubes slanted in an apparatus where the temperature can be slowly raised to between 80° and 90° C. The heating in any case should not exceed 95° C. until the medium is coagulated, or bubbling will occur and the surface will be spoiled. A Koch serum coagulator may be used for this purpose or a small water-oven or even in an Arnold sterilizer, covering the top with a cloth instead of the usual cover. A convenient inspissator we have devised is shown (Fig. 39). It has the advantage that the temperature can be controlled and each tube equally heated. After coagulation the tubes should be sterilized in the Arnold sterilizer for twenty minutes on three successive days. The medium may be coagulated and sterilized in the autoclave by allowing the temperature to rise very slowly until 110° C. is reached. This is rapid and convenient, but it has seemed to us that the high temperature injured the medium somewhat.

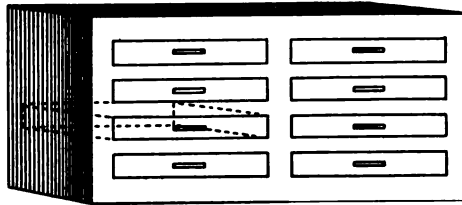


FIG. 39.—Serum inspissator (Park). Ringstands are used for support, and the tilt is easily varied as needed.

The serum is obtained at the slaughter-house in tall cylindrical vessels. These should be disturbed as little as possible until coagulation is complete. If the coagulum adheres to the sides of the vessel, it should be loosened with a glass rod. After twenty-four hours on ice the serum is pipetted or siphoned off. If bloody, it can be placed on ice to allow the corpuscles to settle out.

**Coagulated Blood-serum Media.**—Serum from the cow, horse, sheep, or dog may be coagulated in the form of slants. This type of medium has been used with and without the addition of glycerin for cultivation of tubercle bacilli, but a more satisfactory medium can be prepared from eggs. The serum used should be sterile and, after tubing, heated to 70° C. for one hour. If a clear medium is desired, the best way is to remove a tube from time to time for observation and cease heating when set sufficiently. Higher temperatures or too long an exposure render serum opaque.

Serum may be coagulated in high columns in test-tubes especially for cultivation of spirochetes. The tubes should be warmed to 65° C. and a few at a time placed in water at 75° C. These are tilted every few seconds and removed as soon as they start to set. The heat retained in the tube will complete the coagulation. In this way a soft, almost transparent, coagulum is formed.

The serum may be diluted with saline broth, etc., for special purposes and treated in the same way.

Satisfactory media of the above types require serum free from red cells or dissolved hemoglobin.

**Egg Media.**—Freshly laid eggs are sterile and should be employed for the preparation of media. Egg media are usually coagulated.

**Plain Egg (Dorset).**—The eggs are thoroughly cleansed with water of any adherent dirt and then washed with 5 per cent. carbolic solution and allowed to partially dry. The ends are then gently dried in the flame and pierced with a flamed sharp forceps. The hole at one end should be about  $\frac{1}{8}$  inch in diameter and the membrane broken; the other, which is to be blown into, should be smaller and the membrane left unbroken, if possible. The eggs are then blown into a sterile Erlenmeyer flask, the blowing being done from the cheeks. To the egg is then added 10 per cent. of water by volume of the weight of the eggs. This is mixed by twirling the flask or by gently stirring with a glass rod. Bubbling must be avoided. The mixture is then filtered through cheese-cloth by gravity and tubed. (See Apparatus, Fig. 40,) The tubes are then slanted and coagulated by heating to 70° C. for two or two and a quarter hours on two successive days (see Fig. 39 for apparatus). No further sterilization is employed. The medium is incubated to test its sterility.

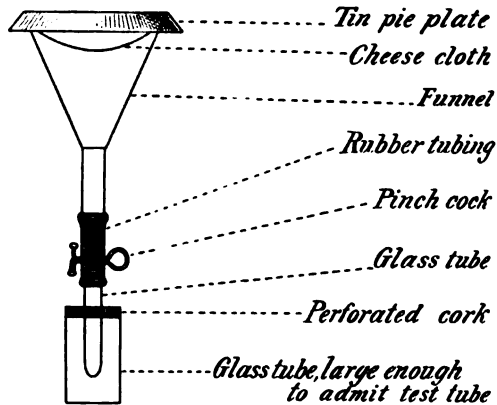


FIG. 40.—Straining and filling apparatus. (Krumwiede after Masson.) The lower tube is plugged with cotton and the top wrapped. It is necessary to loosen the pinch-cock before heating. The whole is sterilised in the autoclave.

**Glycerin Egg (Lubenau).**—Ten eggs are blown into a flask and 200 c.c. of glycerin broth (5 per cent. glycerin, neutral or slightly alkaline to litmus) added. The further preparation is the same as the preceding.

If the media requires it, a drop or two of water can be added after coagulation to supply the necessary moisture. To conserve the moisture the plugs should be paraffined or cut off and burned and a charred cork used to seal the tube. (See also Special Media and Aniline Dyes in Media.)

**Addition of Serum or other Enriching Substances.**—Serum, blood, albuminous body fluids, or tissue extracts containing uncoagulated albumins may be added to media. Usually 1 part to 2 or 3 parts of medium are used. If agar is used, it must be cooled to 50° C. after melting before mixing. If slants are to be made, they should be allowed to set thoroughly, preferably overnight, before raising them, or they may slip down. For certain work it may be necessary to use a 2 per cent. agar to get sufficient stiffness so that the surface is not easily broken; this holds especially for media for plating where surface inoculation is done. Media thus prepared is spoken of as blood agar or broth, ascitic agar or broth, serum agar or broth, etc.

**Watery extracts of tissues,** especially of placenta, are used in the same man-

ner; 500 grams of tissue to a liter of water are used and extracted as for meat infusion, but no heat applied. After preliminary filtration through paper or sand the fluid is sterilized by filtration through a filter candle.

*Nutrose* may be used in media in 1 per cent. amounts to aid growth.

*Serum Media.*—For special purposes serum or other albuminous fluids, such as ascitic fluid, are used. They may be used as such or diluted with saline or by the addition of nutrient media. For these special purposes, sterilization by heat or even filtration should be avoided.

*Serum-water Media.*—If serum is dialyzed to remove the salts, it can be heated to 100° C. without coagulation. Serum diluted with two or three times its volume of distilled water can be sterilized in the Arnold sterilizer. This is so-called "serum-water medium" (Hiss) to which 1 per cent. of sugar is added for fermentation tests. Acid production is shown by the change in the indicator added and by coagulation of the serum. Gas production is shown by the bubbles in the coagulum.

Inulin serum-water medium is the most commonly used preparation of this type. (See under Carbohydrates.)

A similar medium is prepared as follows: Inulin (or other carbohydrate) 4 grams, peptone 4 grams, water, 200 c.c. Add litmus or other indicator. Dissolve and tube (2 or 3 c.c. to a tube) and sterilize. The method depending on the sugar used and precautions already given. After cooling add to each tube an equal quantity of sterile ascitic fluid or serum.

*Blood Media.*—Blood for media is most easily obtained from larger animals by introducing a trocar into the jugular. From smaller animals anesthesia is used and the carotid is dissected out for the introduction of the trocar or cannula. Small quantities of blood may be obtained by heart puncture with a syringe. The steps must be aseptic, as the blood cannot be sterilized. The fluid blood may be mixed with the medium at once or it is defibrinated or mixed with citrate solution when it is to be stored. One part of a 10 per cent. sodium citrate solution is added to 9 parts of blood.

The blood may be mixed with solid or fluid media as already described. For streptococci and its allies a good medium is obtained by placing a drop of blood on the surface of an agar slant. For certain purposes the agar is used in Petri dishes with a drop of blood on the surface. The blood is streaked out during inoculation.

*Hemoglobin Media.*—Certain bacteria, like the influenza bacillus, require hemoglobin for their growth. This may be supplied by the addition of whole blood or by dilutions of the blood which has been laked in distilled water. A final dilution of 1 to 500 still gives a good growth. For agar slants the blood should be mixed with the agar at 95° C. For plate work the mixing should be below 55° C.

*Digestion Products in the Preparation of Media.*—*Martin's Peptone Solution.*—Five pigs' stomachs cleaned, fat removed and finely minced. (A number of stomachs should be used to equalize the peptone content; in this way an almost average composition in peptone is obtained.) Mixed stomachs (minced) 200 grams, hydrochloric acid (pure) 10 grams, water (at 50° C.) 1000 c.c. Keep at a temperature of 50° C. for twenty to twenty-four hours in a glass or porcelain vessel, *not* enamel. It is most important not to allow peptone to come in contact with any metal until it is neutralized. Heat to 80° to stop digestion. Pass through a layer of absorbent cotton. (At this point it can be stored without sterilization.) Heat filtrate to 70° C. and neutralize to litmus at this temperature. Sterilize in autoclave at 10 pounds' pressure for fifteen to twenty minutes and store away.

*Martin's Peptone Broth.*—To meat infusion add an equal amount of Martin's peptone which has been neutralized. The mixture is best made with both the peptone and broth at 70° C. Titrate (room temperature method) and set to desired reaction. Autoclave for fifteen minutes to clear. Titrate again and set reaction as before. Filter through paper and cotton and sterilize in autoclave at 15 pounds' pressure for one-half hour.

**Digested Meat Media.**—Meat, etc., digested by pancreatin, trypsin and other ferments.—Hottinger makes the statement that the greater part of the nutritive elements of meat are lost in the usual process of making broth for laboratory use. To avoid this, a process of slow digestion is recommended, with the addition of pancreatin, and it is claimed that in this way a much more favorable medium can be obtained for the growth of bacteria, with so much peptone developed as a result of the meat digestion, that no addition of commercial peptone is required. Sodium chloride is also omitted.

**Hottinger Broth** (slightly modified).—Use 750 grams of meat to 1500 c.c. water. The meat, carefully freed from fascia, is cut in finger-thick pieces. Heat the water to boiling. Drop in the meat, piece by piece, stirring constantly. Boil up strongly and take from fire. Take out meat and put through chopping machine. Cool water to 37° C. and to it add sodium carbonate, 1.5 grams per liter. Put chopped meat in flasks (2-liter, Erlenmeyer) 550 grams per flask. Add the water (37° C.) to flasks, filling them up to narrow neck. To each flask add: pancreatin 3 grams, chloroform 10 c.c., toluol 10 c.c.

Cork tightly and shake well. Incubate at 37° C. overnight. Shake next day and add more pancreatin unless the fluid shows a yellow color and particles of meat look smaller, showing that digestion is taking place.

The process of digestion should continue for four or five days at room temperature or for two or three days in incubator, shaking well each day. At the end of this time the meat has become a finely divided mass, giving off a very offensive odor. According to Hottinger the medium may be stored in the ice-box at this point without heating again, after testing with litmus paper and acidifying with a slight amount of dilute HCl, if found to be alkaline. In our experience this plan has not worked well, and we have found it best to proceed at once as follows:

Decant liquid through cheese-cloth. Add an equal amount of water to the residue in flask. Shake well. Allow meat to settle and again decant. Finally place meat on cheese-cloth and allow to drain. Boil the filtrate for a few minutes, then filter through absorbent cotton and paper until clear. Store in flasks as stock broth after autoclaving at 15 pounds' pressure for one-half hour.

This stock can be diluted for use as desired, according to Hottinger ten, twenty or more times. Diluted one-half (1 part stock broth, 1 part water) this broth gives excellent results with the ordinary laboratory organisms. The diphtheria bacillus, however, does not grow well.

**Hottinger Agar.**—Add sufficient water to Hottinger stock broth to make the required dilution. Add agar 1.5 per cent. and proceed as usual.

This agar made with Hottinger broth, 1 to 1 dilution, gives good results in successive slant agar cultures of the usual laboratory organisms with the exception of the gonococcus and the diphtheria bacillus.

**Broth Prepared with Trypsin.**—Use 300 to 500 grams of meat to 1 liter of water to which 0.4 per cent. sodium carbonate has been added. Soak overnight, then heat to 80° C. Cool to 38° C. and add 15 c.c. of liquid trypsin. Keep at 38° C. for five hours, stirring frequently. If kept overnight at this temperature, add toluol 10 c.c. (or thymol crystals). Then add hydrochloric acid (normal) to neutralize. Boil seven minutes, strain, set reaction. Boil one-half hour, filter and sterilize.

**Dilutions of Media.**—Berry has found in milk plates that good results are obtained with a medium containing the usual amount of agar and lessened amounts of meat extract, peptone and salt. With the progressive dilution of the medium; there is, at first, an increase in the number of colonies, but beyond a certain point they tend to become smaller and less distinct. On agar made with a dilution of one-twelfth the usual amount of meat extract, peptone and salt, the colonies are practically identical in number and appearance with those on the standard agar.

Still better results for milk work are given by agar made with a 1 to 15 dilution of Hottinger stock broth.

**Special Media.**—*Bordet-Gengou.*—Water 1000 c.c., glycerin 40 c.c., potatoes (sliced) 500 grams. Heat in an autoclave at 15 pounds' pressure for a half-hour. Pour off the liquid. To 500 c.c. of this potato extract add salt solution (0.6 per cent.) 1500 c.c., and agar 60 grams. Autoclave to dissolve, filter and tube. When used, an equal quantity or less of defibrinated blood is added.

*Liver Agar* (recommended by Dopter, Penfold and others).—Substitute minced liver for meat and proceed as in preparing infusion agar. With a reaction of 0.2 per cent. to phenolphthalein it is especially adapted for meningococcus.

*Starch Medium.*—(Vedder).—Beef 500 grams, water 1000 c.c., extract in ice-chest overnight, boil and strain, add agar 1.5 per cent. and dissolve. Correct reaction to 0.2 per cent. to 0.7 per cent.+. Cool and clarify with eggs. Filter, add 1 per cent. of starch and heat in Arnold for forty-five minutes, shaking the medium several times to distribute the starch. Tube and autoclave fifteen minutes at 10 pounds' pressure. Recommended especially for meningococcus.

*Use of Fresh Tissue in Media.*—Sterile fresh tissue may be added to media. This not only adds nutrient material but aids the production of anaërobic conditions. Smith first used it in fermentation tubes for growing anaërobes, Williams uses it on agar for the growth of pure cultures of amebæ, and Noguchi uses it in special media for spirochetes and other feebly growing organisms. In the latter medium rabbit's kidney is most commonly used. Small pieces are added to fluid media or placed in test-tubes and serum or ascitic agar added. In the case of coagulated serum the tissue is pushed to the bottom of the tube.

*Media Heated after the Addition of Tissues, etc.*—Especially good for the pathogenic (proteolytic) anaërobes. Drop a piece of meat into gelatin or broth tubes and sterilize, or cook the meat in water, alkalize, sterilize and tube; e. g., bullock's heart minced finely and then ground in a mortar, 8 ounces to tap-water 8 ounces.

Egg protein may also be used. Add one egg to 300 c.c. of water, mix, and bring to boil slowly, shaking frequently, tube and sterilize. An alkaline-egg mixture, yolk of one egg and whites of two, to 500 c.c. of water and 6 c.c. of  $\frac{1}{2}$  NaOH may be used (as under Cholera Media) in proportion of 1 to 5 in broth or agar. (Robertson.) See also milk, and inspissated serum or egg media for other specially suitable media.

**Synthetic Media.**—For certain work it is an advantage to know the exact chemical constitution of the medium. Then, too, some bacteria, especially certain species in soil, refuse to grow on the more complex media. Pigment production is very easily observed on these media.

*Uchinsky's (Fränkel's Modification).*—Water 1000 c.c., asparagin 4 grams, ammonium lactate 6 grams,  $\text{Na}_2\text{HPO}_4$  2 grams, NaCl 5 grams.

*Modified Formula for Disinfection Tests.*—Water 1000 c.c., asparagin 6 grams, sodium phosphate (ortho) 2 grams, sodium chloride 5 grams. Dissolve and if necessary render alkaline to litmus by addition of NaOH. Sterilize in small tubes and test for color production with *B. pyocyaneus*.

*Ringer's Solution.*—Sodium chloride 10 grams, potassium chloride 0.2 gram, calcium chloride 0.2 gram, sodium bicarbonate 0.1 gram, glucose 1 gram, water 1000 c.c.

For broth add 1 or 2 per cent. peptone.

For agar add 1.5 or 2 per cent. agar and 1 or 2 per cent. peptone.

Dissolve, clear, and filter.

**Use of Aniline Dyes in Media.**—Various basic aniline dyes show a differential restraining action on the growth of bacteria. Gentian violet and allied dyes when present in a dilution of 1 to 100,000 preferably in agar, inhibits the growth of the Gram-positive group of bacteria but has no effect on the growth of the Gram-negative bacteria. This is a general rule. Gentian violet may be employed to prevent growth of Gram-positive germs or for isolation of Gram-negative germs from contaminated material where the contamination is Gram-positive. An example of this is the use of crystal violet in Conradi medium. Exceptions to the above general rule occur, thus the acid-fast but Gram-positive group grow freely even when gentian violet

in strong concentrations is used. Because of this, pure cultures of acid-fast bacilli (*B. tuberculosis*) can be obtained by this means from contaminated material, as sputum or feces.

**Gentian-violet-egg Medium** (Petroff).—Meat extract (veal or beef 500 grams to 500 c.c. of 15 per cent. glycerin, extract twenty-four hours, and collect fluid by means of press; sterilize by filtration). Add 1 c.c. of a 1 per cent. alcoholic solution of gentian violet to each 100 c.c. of meat extract, mix with 2 parts of whole egg, tube, inspissate on three days for three-quarters of an hour at 80° to 85° C. This medium is especially recommended for the isolation of *B. tuberculosis*.

Certain dyes have a differential action on closely allied bacteria inhibiting completely or partially the growth of one, but allowing another to grow freely. An example of this is the action of brilliant green on the typhoid, paratyphoid, and colon bacillus. The colon types are completely or nearly completely restrained, typhoid less so, and paratyphoid least of all. This is applied in isolation of typhoid or paratyphoid from stools. (See below.)

The use of dyes as indicators has been already noted.

**Special Media for Typhoid, Paratyphoid, Dysentery, and Colon.**—Numerous media are used. The most commonly employed plating media are Endo and Conradi-Drigalsky.

**Endo Medium** (Kendall's Modification).—The basis is ordinary meat-extract agar, slightly alkaline to litmus. This should be sterilized in bottles in 100 c.c. amounts. When needed, 1 gram of lactose is poured into a bottle and the agar melted which dissolves and sterilizes the lactose. To each bottle, after melting, is added 1 c.c. of decolorized fuchsin prepared as follows: To 10 c.c. of a freshly prepared 10 per cent. watery solution of sodium sulphite add 1 c.c. of a saturated alcoholic solution of fuchsin and heat in the Arnold sterilizer for twenty minutes. Plates are then poured and allowed to harden without the covers, and dried in the incubator for thirty minutes, protecting the plates from dust. The medium must be mixed each time when needed and the plates used. The color returns gradually and the plates are then useless. (See also under Media for Water Examinations.)

**Robinson and Rettger's Modification of Endo's Medium.**—This medium has given sharper differentiation than the above medium.

Preparation of agar: Water, 1000 c.c.; agar, 25 grams; peptone (Fairchild's), 10 grams; meat extract, 5 grams. Dissolve the agar, meat extract, and peptone. Make neutral to litmus, autoclave for thirty minutes at 15 pounds' pressure. Filter through cotton and cheese-cloth. Add 10 c.c. of a 10 per cent. sodium carbonate solution; heat for ten minutes; add 1 per cent. lactose, 5 c.c. saturated alcoholic fuchsin, and finally 10 c.c. of a 10 per cent. solution of anhydrous sodium bisulphite. The medium is now tubed in 20 c.c. amounts and autoclaved for seven minutes at 10 pounds' pressure. The best results are obtained when the reaction of the medium is brought, after the addition of the lactose and sodium bisulphite, before adding the fuchsin, to +0.1 phenolphthalein, hot titration.

According to our experience it seems better to prepare the medium, adjust the reaction, bottle in 100 c.c. amounts, and add the lactose, fuchsin, and sodium bisulphite just before use.

**Conradi-Drigalsky Medium.**—Water 1000 c.c., agar 20 grams, sodium chloride 5 grams, peptone 20 grams, nutrose 10 grams, beef extract (Liebig's) 4 grams, normal NaOH 50 c.c. Dissolve the ingredients in an autoclave, cool, and clear with eggs. Adjust reaction to a moderate but distinct alkalinity to litmus. To each liter of agar thus prepared are added 130 c.c. of Kubel and Thiemann litmus solution, crystal violet (1 to 1000 solution) 10 c.c., and 15 grams of lactose. Heat in an Arnold sterilizer ten minutes to obtain thorough mixing and fill in tubes or bottles and sterilize in Arnold sterilizer. Omit the crystal violet if to be used for dysentery.

**Russell's Double Sugar Medium.**—To ordinary extract agar adjusted neutral to litmus add 1 per cent. of lactose and 0.1 per cent. of glucose and sufficient

litmus to give a good color. Or add 1 per cent. of Andrade indicator in place of litmus and adjust reaction to this indicator. Tube and slant, leaving a generous "butt" at bottom of tube for stab inoculation.

**Brilliant Green Agar (Krumwiede) for Isolation of *B. Typhosus* and *B. Paratyphosus*.**—The basis of the medium is prepared as follows:

A. Water 1 liter, agar 30 grams; autoclave into solution.

B. Water 1 liter, meat extract 6 grams, salt 10 grams, peptone 20 grams; Arnold until dissolved.

Mix A and B; add normal soda (see below); boil one-half hour. Cool, clear with egg, filter until clear, bottle and autoclave. The agar, when used, must be neutral to Andrade's indicator (*q. v.*); it is convenient to adjust it at the time of preparation. For trial, 8 c.c. of soda per liter are added before clearing, and the agar finished. At the time of use, the reaction is tested, and if not found satisfactory, adjusted; allowance is made in subsequent batches so that, with the same brand of peptone, it is possible to obtain a constant and uniform reaction without titration. We find it convenient, on account of the necessity of standardizing the agar, to prepare large batches (20 liters); if this is done, the entire bulk of agar intended for use in brilliant green medium must be well mixed in one container after filtering. At the time the medium is to be used, a number of bottles are melted, and to each 100 c.c. is added 1 c.c. of indicator (Andrade's), 5 c.c. of a sterile solution in distilled water of 20 per cent. of lactose and 2 per cent. of glucose (this will give 1 per cent. lactose and 0.1 per cent. glucose in the agar), and finally the appropriate amount (see below) of a 0.1 per cent. solution of brilliant green in distilled water. The medium is well mixed and poured into rather thick plates (no more than six from 100 c.c.). Porous tops are convenient, as dry plates are essential.

The selective bactericidal action of brilliant green is exhibited only at certain high dilutions. It is quantitative and varies according to the medium and the material inoculated. Even with similarly prepared batches of agar, different amounts of dye may be required to obtain the same restraining action. It is therefore necessary to determine for each batch the optimal dilutions for use, and these dilutions and the same dye solution are used for the rest of that preparation.

The method of standardization is to pour plates of four dilutions of dye: 1 to 500,000, 1 to 330,000, 1 to 250,000, 1 to 200,000, which correspond to 0.2, 0.3, 0.4, 0.5 c.c. of 0.1 per cent. dye solution to 100 c.c. of agar. These plates are evenly inoculated with broth cultures of typhoid, paratyphoid A and B, which have been diluted to give 75 to 200 organisms per loopful (loop of broth culture to 15 c.c. of broth), and when possible, with stools from typhoid carriers or cases, or with dilutions of broth cultures in suspensions of normal stools. A plate of each dilution and a control plate of a non-restraining medium, such as Endo's, is used for each culture or stool.

These appropriate dilutions are (1) the lowest dilution at which the typhoid colonies are of good size and undiminished in number as compared with the control plate, but many fecal types are excluded; (2) a lower dilution, where the typhoid colonies are slightly reduced in size and number, but almost all the other flora have disappeared, the degree varying with different stools. They correspond, in general, to the effect we obtain with 0.2 and 0.3 c.c. respectively. For paratyphoid, the dye can usually be safely used in lower dilutions: 1 to 200,000. In investigating an outbreak of paratyphoid the medium can be standardized directly against the offending strain as soon as one is isolated. We have found two dilutions preferable for routine examinations, as the fecal proteins, which vary in amount in different suspensions, reduce somewhat the activity of the dye; and, moreover, the fecal flora of different stools as well as various strains of typhoid vary in their dye sensitiveness. With two concentrations these variables are balanced. For an occasional examination with unstandardized materials, 0.2, 0.3, 0.4 (0.5 if paratyphoid is suspected) will probably cover the range of variation.

To inoculate, the stool is emulsified (if fluid, it may be slightly diluted) in



about 15 volumes of saline or peptone and the heavier particles are allowed to sediment for one-half hour. For routine work, two strong, two weak and two Endo plates are used for each sample; one loopful of suspension is placed on a weak dye plate, the same on a strong plate; these are evenly smeared with a heavy platinum or nichrome spreader, which is then carried over to an Endo plate which has received no direct material; a second series of weak and strong dye plates is inoculated with twice this amount, and spread in the same way to an Endo plate. The spatulum is not flamed between plates. This will usually give satisfactory seeding of Endo as well as dye plates, although the former receive only the material carried over on the spreader. If stronger dye is used, as for paratyphoid, more material may be inoculated.

After eighteen hours' incubation the typhoid colonies are characteristic in appearance. They are of good size (1 or 1.5 mm.), as the glucose favors their growth. Viewed through the plate against a dark background, with the light passing obliquely through the agar, they have a peculiar marking; with artificial light and hand lens, under the same conditions, they have the appearance of a coarse wool fabric. The colony may have a slight pinkish tinge from fermentation of the trace of glucose; this renders it still more distinctive. The larger colonies resemble paratyphoid bacillus; they are heavier, and more opaque, the markings are less evident, and they may be tinted a delicate green. Paratyphoid A resembles the typhoid colony more nearly than the paratyphoid B; it is frequently extremely flattened, slightly tinged, and has an ill-defined edge. All the types show better markings on stronger dye. On weak dye plates (rarely on strong) there may develop typhoid-like colonies of coliform organisms, but their exaggerated striations, with distinct crossbars, usually exclude them. Many of these typhoid-like organisms agglutinate spontaneously on the slide when the macroscopic slide technic is used for identification (see Agglutination).

**Fluid Media Containing Brilliant Green.**—Browning, Gilmour and Mackie, Krumwiede and Pratt, Torrey, Robinson and Rettger and many English workers have successfully used brilliant green in peptone-water or broth, sometimes with the addition of glucose, for the isolation of typhoid or paratyphoid bacilli from feces. It is essential to use graded dilutions of the dye; dilutions from 1 to 100,000 to 1 to 500,000 are generally advised. The medium is sterilized in 5 or 10 c.c. quantities in test-tubes, and the dye added just before use; the reaction is set neutral to phenolphthalein (Robinson and Rettger advise 1 per cent. acid). After twelve to eighteen hours the highest concentration tubes showing growth are plated on Endo's medium. While the pathogenic organisms are frequently lost in this medium by overgrowth of mucoid *aërogenes* types, enrichment in brilliant green peptone-water or broth may give positive results where even brilliant green agar fails; for routine work, the above fluid media add too much to the time, cost and labor of examination to warrant their use except in special instances. Teague and Clurman have recently reported a medium which they claim gives better results.

**Special Media for Cholera.**—*Peptone Solution* (see p. 98).

*Saccharose Peptone-water* (Bendick).—To 1000 c.c. peptone solution, neutralized to phenolphthalein add 1 gram of anhydrous sodium carbonate. Boil and filter. Add 5 grams of saccharose and 5 c.c. of a saturated solution of phenolphthalein in 50 per cent. alcohol. Tube and sterilize in Arnold sterilizer.

*Dieudonné's Medium.*—Mix equal parts of defibrinated beef blood and normal sodium or potassium hydrate solution and steam in the Arnold sterilizer for a half-hour; 3 parts of this are added to 7 parts of 3 per cent. agar (neutral to litmus) and poured into Petri dishes (15 c.c. to a 10 cm. dish). Let them harden uncovered, but protected by paper. Place strips of filter paper between the dish and cover to aid in the absorption of the moisture and ammonia and place in the incubator for twelve to fifteen hours. Nothing will grow on the medium when first made. The plates are good for about ten to fourteen days. Various modifications of this medium have been suggested.

**Pilon.**—Substitute 12 per cent. sodium carbonate (crystalline) solution for the sodium hydrate. The plates can be used after thirty minutes' drying.

**Alkaline-egg Medium** (Krumwiede).—Make an egg-water mixture, using equal parts of egg and water. Mix equal parts of the egg-water and 12 per cent. sodium carbonate (crystalline) and steam in the Arnold sterilizer for twenty minutes. Mix while hot 30 parts to 70 parts of 3 per cent. agar (the meat extract may be omitted) and pour into plates and dry for twenty minutes. If the meat extract is omitted, the colonies are small but very characteristic. The colonies are much increased in size when extract is present or if 0.5 per cent. of glucose or saccharose is added. The reaction of the agar need not be corrected. Goldberger claims that the alkalinity is unnecessarily high, and makes the egg mixture as given with a 6.5 per cent. solution of anhydrous sodium carbonate and mixes 1 part of the egg with 5 parts of glucose extract agar.

**Media for Milk, Water, Shell-fish, etc., Standard Methods.**<sup>1</sup>—**Water.**—The basis for broth, gelatin or agar is 3 grams of beef extract (Liebig's or its equivalent by tests) and 5 grams of peptone (Witte's or equivalent) to the liter. Gelatin media should contain 10 per cent. and agar media 1.5 per cent. of the respective substances. The agar should be soaked in water to rid it of salts. Both agar and gelatin are dried at 105° C. for one-half hour before weighing. If the reaction of the medium is not already between +0.5 and +1, adjust to +1.<sup>2</sup> Sugar broths are prepared by the addition of 1 per cent. of the required carbohydrate. The reaction of sugar broth should be neutral to phenolphthalein. Litmus (reagent of highest purity) should be in 2 per cent. solution. Azolitmin (Kahlbaum), 1 per cent. solution, may be used as a substitute. Adjustment of reaction of the solution may be necessary. One cubic centimeter of litmus solution is added to 10 c.c. of lactose agar for litmus-lactose-agar plates when poured. All media and litmus solutions are to be tubed before sterilization and sterilized at 15 pounds' pressure for fifteen minutes, quickly removed and cooled rapidly. The Endo recommended has as a basis an agar containing 3 per cent. of agar, 1 per cent. of peptone and 0.5 per cent. of Liebig's extract. The medium is sterilized in 100 c.c. lots. Just before use add to the melted agar 1 gram of lactose and 0.5 c.c. of a mixture of 10 c.c. of a 10 per cent. solution of sodium sulphite and 2 c.c. of a 10 per cent. solution of basic fuchsin in 95 per cent. alcohol. (See under Media for Typhoid.) For presumptive tests for *B. coli*, lactose broth in fermentation tubes is to be used.

**Milk.**—The present standard agar contains: Agar 1.5 per cent., beef extract (Liebig's) 0.3 per cent., peptone 1 per cent. No sodium chloride.

**Shell-fish.**—Use agar as for water for count. Lactose bile (see below) is still employed.

**Other Media for Water, Sewage, Shell-fish, etc., Examinations.**—**Neutral Red Lactose Peptone.**—To peptone solution add 1 per cent. of lactose and 1 per cent. of a saturated aqueous solution of neutral red. Tube in fermentation tubes.

**Liver Broth.**—Beef liver 500 grams, peptone 10 grams, dextrose 10 grams, dipotassium phosphate ( $K_2HPO_4$ ) 1 gram, water 1000 c.c. The liver is boiled for two hours for extraction and strained. Then dissolve other ingredients and adjust reaction.

**Lactose Bile.**—Add 1 per cent. of peptone and 1 per cent. of lactose to ox bile. Tube in fermentation tubes and sterilize.

**Special Media for Toxin Production, etc.**—**For Diphtheria Toxin** (Banzhaf).—Lean veal free from fibrous tissues, minced, 1 pound to the liter of water. Soak overnight and then heat to between 45° and 55° C. for one hour. Then bring to a boil. Strain. To fluid add peptone (Witte) 1.5 per cent. and sodium chloride 0.5 per cent. Boil for a half-hour. Adjust reaction to 1.2 per cent. acid to phenolphthalein. Place about 800 c.c. in 2-liter Erlenmeyer flasks and

<sup>1</sup> See Am. Jour. Public Health, 1917, for final reports of present committees.

<sup>2</sup> Boiling for one minute advised in titration (see p. 90).

add 3 grams of calcium carbonate. Sterilize in an autoclave at 15 pounds' pressure for a half-hour.

**Diphtheria Toxin Broth** (Martin's Peptone).—Lean veal (market) free from fibrous tissue, minced, 1 pound to the liter of water. Incubate for eighteen to twenty-four hours at 35° C. Heat at 45° to 48° C. for one hour. Boil briskly for a half-hour. Strain through cheese-cloth. Measure the filtrate and warm it to 70° C. Add an equal amount of Martin's peptone (p. 103) which has just been neutralized at 70° C. A clear product usually results from mixing them at this temperature. Heat to boiling. Titrate (room-temperature method) to +0.5 (phenolphthalein). Autoclave at about 10 pounds' pressure for fifteen to twenty minutes to clear. Titrate again and adjust to +0.5 as before if necessary. Filter through cotton and paper into flasks, allowing 800 c.c. to a flask. Sterilize one-half hour at 15 pounds' pressure.

**For Tetanus Toxin.**—One pound of lean veal per liter of water. Soak overnight in ice-chest. Heat to 45° C. for one hour, then boil for one-half hour. Strain, add peptone (Witte) 1 per cent., salt 0.5 per cent., and glucose 1 per cent. Boil until ingredients are melted, and adjust reaction to +1 per cent. Sterilize in 2-liter Erlenmeyer flasks, in Arnold sterilizer, leaving only sufficient space in flask for expansion during heating. Heat for one and a half hours on first day and for one hour on second day.

**Tetanus Toxin Broth** (Martin's Peptone).—Lean veal (market) free from fibrinous tissue, minced, 1 pound to the liter of water. Soak in ice-box for eighteen to twenty-four hours. Heat to 45° to 48° C. for one hour. Boil briskly for one-half hour. Strain through cheese-cloth. Measure the filtrate and warm to 70° C. Add an equal amount of Martin's peptone which has just been neutralized at 70° C. The two liquids should be at the same temperature, for then a clear product usually results. Heat to boiling. Titrate (room-temperature method) to +1 (phenolphthalein). Autoclave at about 10 pounds' pressure to clear. Measure and add 1 per cent. glucose (powder). Boil two or three minutes. Titrate again and set reaction as before to +1. Filter through cotton and paper directly into 2-liter Erlenmeyer flasks, leaving only sufficient space in flasks for expansion of broth during sterilization in the Arnold. Sterilize flasks for one and a half hours on the first day and one hour on second day.



FIG. 41. — Platinum needle and loop. For most purposes finer wire is used.

**Mallein Broth** (Eye and Subcutaneous).—Lean veal free from fascia, minced, 1 pound to the liter of water. Macerate overnight at room temperature. Heat at 45° to 50° C. for one hour. Boil up strongly. Strain through cheese-cloth. Add peptone (Witte) 1 per cent., sodium chloride 0.5 per cent. Boil again to dissolve the peptone. Titrate (room-temperature method) and adjust reaction to +2.5 (phenolphthalein). Autoclave for fifteen minutes at 15 pounds' pressure to clear. Filter through cotton and paper. Measure and add glycerin, 5 per cent. Put 250 c.c. broth in each quart Blake bottle or liter flask. Sterilize in autoclave one-half hour at 15 pounds' pressure.

**Special Media for Yeasts and Molds.**—Most of the pathogenic yeasts and molds can be cultivated on the media already described. Growth is much improved if glucose or maltose is added. The reaction should, however, be acid or at most slightly alkaline to litmus.

For molds, especially those infecting the skin, an agar containing no meat but 1 to 2 per cent. peptone and 2 per cent. of glucose or maltose and 0.5 per cent. of glycerin with no change of reaction (*Sabouraud*) gives a very favorable medium.

For yeasts, beerwort media are especially favorable. Hopped beerwort is obtained at a brewery. Autoclave, cool, and filter, and then tube (this avoids subsequent precipitation). For solid media add 10 per cent. gelatin or 1.5 per cent. agar. Various carbohydrates may be added in 2 per cent. amounts.

**Special Media for Protozoa.**—*For Blood Flagellates* (Novy and MacNeal).—Equal parts of nutrient agar and fresh defibrinated blood (rabbit or rat). The medium is allowed to stiffen slanted, so more water of condensation may gather at bottom of slanted surface. The medium should be planted while fresh with blood or other infected material containing the living flagellates (e. g., trypanosomes, Leishmania).

*Medium for Malaria Organisms* (Bass and Johns).—10 c.c. of blood drawn from a malarial patient, and carefully defibrinated. This is placed in smaller test-tubes in 1 c.c. amounts. One per cent. of a 50 per cent. solution of dextrose is added to each small test-tube before adding the blood. The red corpuscles settle so that a  $\frac{1}{2}$  cm. layer of serum is left about them. The parasites grow in a thin layer near the top of the cell sediments. Beneath this zone the parasites die. Bass says leukocytes should be gotten rid of because they destroy the parasites, but the Thompsons say this is unnecessary. Most favorable temperature 39° to 40° C.

*Ameba Agar* (Musgrave and Clegg).—Agar 1 per cent., tap-water 90 per cent., ordinary nutrient broth 10 per cent. Mix and sterilize as usual. Reaction about neutral to phenolphthalein.

*Medium for Pure Cultures of Amebæ* (Williams).—Fresh sterile brain, liver, or kidney is cut in small pieces and placed on the surface of ameba agar.

**Used Agar.**—It has been found by Berry of this laboratory that veal agar which had been used in Blake bottles for preparation of antigens and vaccines in large quantities, can be used a second time for culture purposes. After the surface growth is removed by scraping and washing with salt solution or distilled water, the agar is melted and the different lots poured together. The agar is then made neutral to phenolphthalein, tubed, sterilized and again planted with various organisms.

Except for a loss of transparency, which could probably be restored by fresh clearing and filtering, the previous use of the agar seems in no way to affect its value as a culture medium. Typhoid, paratyphoid, coli, staphylococcus, streptococcus, pneumococcus, gonococcus, diphtheria and other varieties of ordinary laboratory organisms grow as vigorously as on new agar, and in some cases the growth is heavier. Abundant cultures of amebæ are also obtained on used agar.

## CULTIVATION OF MICROÖRGANISMS.

Microorganisms can seldom be identified by the microscope alone. By this method certain characteristics, such as individual form, arrangement, individual reaction to stain, and motility or lack of motility can be studied. But in order to learn all the characteristics of an organism, including its specific relation to disease, we must be able to grow it in pure culture, that is, apart from all other organisms, and determine its cultural characteristics on various media, its immunity reactions and its action in the animal body. Koch's postulates (see p. 23) were based on the ability to obtain pure cultures. In cultivating microorganisms it is not only necessary to supply the appropriate foodstuff, but also to have the appropriate conditions as to temperature, moisture, access of oxygen, etc.

When we make cultures of any material, we are likely to find that instead of only one variety of microorganism, there are a number

present. If we grow the material in *fluid media*, we find that as the different varieties develop they spread through the medium and become hopelessly mixed; furthermore, the more vigorous varieties outgrow the feebler ones, which are thus lost. If, however, the microorganisms in the material be scattered through or on a *solid medium*, they may grow in separate colonies which may be visible to the naked eye or can be seen with a low-power lens. From each of these colonies a pure culture may be obtained.

**Methods of Isolating Pure Cultures.**—*Plating Methods.*—Two plating methods are employed: pour plates or streak plates. Agar or gelatin media are used. The medium is first rendered fluid: gelatin, by placing the tubes in warm water (35° to 40° C.); agar, by immersing the tubes in boiling water until fluid and then cooling the tubes to between 40° and 42° C. Agar only melts completely when heated to the boiling-point of water or thereabouts and hardens as the temperature falls below 40° C. The cooling of the agar is necessary, otherwise the organisms would be injured by the heat. If enriching fluids are to be used, they are added to the agar when between 45° and 50° C. The material from which we wish to obtain pure cultures is then inoculated into the fluidified media. The method of inoculation is in general as follows: A loop of the material is carried into a tube of the medium and thoroughly mixed with the medium by tilting and rolling the tube. Agitation sufficient to produce bubbles must be avoided. From this tube three loops are carried over to a second tube which is mixed in the same manner. From the second tube five loops are carried to a third tube and mixed. The lips of the tubes are flamed and the contents of each poured into a Petri dish, which is, if necessary, tilted to spread the agar evenly over the bottom of the dish. In pouring, the lid of the dish is raised just sufficiently to pour the contents of the tube into the dish, the lid acting as a protection against falling dust. In diluting the material with culture media, it is our object to separate the organisms so that when colonies develop from them they will be well separated, that is, *discrete*. If they were not well separated, we would touch several colonies in attempting to transfer the growth of one and thus fail to obtain pure cultures. As we have no way of telling what dilution will give us well-separated colonies, a series of dilutions is necessary. The method will have to vary with the probable number of organisms in the material. If they are in great numbers, further dilutions of the material will be necessary, as the first tube will give overcrowded plates. A preliminary dilution or two may be made in saline solution or broth. When the material contains very few organisms, more material must be inoculated into the first tube. The dilutions may be made with a sterile pipette instead of a loop. The tip of the pipette is touched to the material and carried over to the first tube of medium, mixing being done by sucking the medium up and down in the pipette; about 0.1 c.c. of the contents of this tube is carried to a second tube, 0.1 c.c. of this to a third, etc.

*Streak Plates.*—In this method the medium (agar) is poured into Petri dishes and allowed to harden. The material is then streaked over the surface of the medium either with a platinum loop or spatula or a bent-glass rod. Two or more plates are inoculated in succession without further sterilization of the loop or spatula. This dilutes the material so that discrete colonies are obtained. A difficulty that is likely to arise in the use of streak plates, and to a less degree with pour plates, is collection of water of condensation, which, finding its way to the surface of the plate, will cause a spreading of the growth over the whole surface. This can be avoided to a large extent by cooling the agar before pouring on the plates. Special covers made of porous earthenware can be obtained, which will absorb the moisture. Incubating the plates upside down is a good precaution. Motile bacteria are most likely to spread over the surface of media. The special media used for the isolation of fecal

bacteria must, because of the great numbers of motile bacteria in the stool, be comparatively dry. As most of these media restrain the ordinary contaminating bacteria from growing, they may be allowed to harden and dry in the Petri dishes with the covers off, and the drying continued by placing them in the incubator partly open.

**Fishing Colonies.**—This is the term used for the process of transferring the microorganisms from the colony to a fresh medium. This is usually done with a straight platinum wire, which is less likely to touch any colony other than the one we wish to fish. Fishing is done either by selecting the colony with the unaided eye or by the aid of a microscope. The advantage gained by the use of the microscope is that we can be sure that the colony to be fished has no microscopic colonies in the immediate neighborhood likely to be touched by the platinum wire. Furthermore, if the fishing is done under the microscope, the fact that one and only one colony has been touched is visually proved. Opaque media are fished by eye. In fishing under the microscope the colony to be fished is centred under a No. 2 or AA objective and the wire introduced between the objective and the agar until the tip of the wire touches the colony. The progress of the wire is watched through the microscope to be sure that nothing is touched but the colony, and to observe by the broken appearance of the colony that it has been touched. The sense of touch is relied on to tell whether the objective is touched. In fishing by eye the plate is held against the light and the colony touched with the wire. In either case the organism is transferred to a fresh medium—usually an agar slant—by rubbing the tip of the wire over the surface of the medium.

**Possible Sources of Error in Plating Methods.**—Theoretically, each colony is the progeny of one single organism, or in the case of organisms that adhere to form pairs, chains, etc., the progeny of such an aggregate of one variety of organism. Practically it not infrequently happens that dissimilar varieties cling together and a colony develops containing both. A second plating from such a colony is usually sufficient to yield pure cultures. In exceptional instances there is the greatest difficulty in separating such “clingers” and it is sometimes impossible.

**Special Methods of Isolation.**—*Dilution Method.*—It is conceivable by gradual dilution of material to reach a point at which some of the loops of the material may only contain one organism which when carried to tubes of medium will yield pure cultures. This method was employed before the introduction of solid media. The uncertainty of the method is evident. For certain purposes modified dilutions are still employed, starting usually with cultures obtained from colonies on plates, the method being employed not so much to obtain pure cultures as to obtain cultures known to be the progeny of one cell.

*Hansen's Method for Yeasts.*—Cover-slips are given a thin coating of paraffin, through which lines are drawn dividing the cover into small squares and each numbered. The marking is done with a needle. The covers are now exposed to hydrofluoric acid to etch the markings into the glass. They are then cleaned and sterilized. Dilute the yeast-containing material with fluidified gelatin until one loop averages one yeast cell as determined by microscopic examination. The gelatin is then spread over the surface of the sterile cover-slip, hardened, and inverted over a Böttcher moist chamber, sealing with vaselin. This is placed under the microscope and the position of the single cells determined, making a sketch for later reference. Incubate at room temperature and when colonies have developed fish those developing from single cells. The advantage of this method over plating is that on plates a proportion of the colonies will develop from adhering cells. The purity of yeasts in industries is of enormous financial importance.

*India-ink Method (Burri).*—The use of india-ink for microscopic examination has already been described. By making successive dilutions of small amounts of culture in drops of ink it is possible to make small spots of ink containing only one organism. This is determined microscopically. This is the starting-point for the pure culture from one cell. The reader is referred to

the article by Burri for the technical details. The method has only a very limited application (see page 74).

*Capillary Method (Barber and others).*—Reference is made to the original articles for the details of this method for isolating one cell.

*Preliminary Enrichment Methods.*—Very often it is impossible to isolate certain organisms from material because they are too few in number. With certain organisms it is possible to inoculate the material in special media which will allow free multiplication of this organism, but be unfavorable for the others contained in the material. Examples of this are the use of Löffler's medium for mixed cultures from the throat to find diphtheria, or the peptone method for the enrichment of cholera and other vibrios. Another method is the use of ascitic broth for the isolation of diphtheria from mixed material. The last two methods take advantage of the surface growth of the bacteria. The same end is attained by the use of the following factors: blood media for hemoglobinophilic bacilli; increase of the alkalinity, as in selective media for vibrios; increased acidity, as with tartaric acid, for yeasts; dyestuffs, gentian violet, to inhibit Gram-positive bacteria; brilliant green to inhibit the colon types in mixed cultures from stools; use of heat, spore-containing mixed cultures being heated to 80° C., to kill the non-spore-bearing types; motility, the motile organism spreading from the point of inoculation of the mixed material, as with amebæ and spirochete-containing material; filtration through filters, spirochetes passing filters, most bacteria being held back (see Filtrable Viruses); germicidal agents (see Aniline Dyes, p. 105); antiformin for separation of acid-fast from other bacteria (see under Tuberculosis); finally, the use of animals (see p. 127).

*Special Methods for Anaerobes.*—(See Anaerobic Methods.)

*Applications of the Plating Methods.—Study of Colony.*—The appearance of the colony is one of the points used in identifying bacteria; or the colony appearance being known, the isolation of a certain organism is greatly simplified. In plates showing two or more types of colonies it is an aid in determining the relative proportion of the subsequently identified varieties in the material examined.

In examining colonies both the macroscopic and microscopic characteristics are determined. The age at which a colony will be most characteristic depends on the rapidity of growth of the organism. Daily observations are most suited for full information. As a rule young colonies are most characteristic, although such characteristics as pigment production and gelatin liquefaction may only appear on longer incubation. With the naked eye the following points should be observed: moist or dry, transparent, translucent or opaque, edge sharply defined or indefinite, regular or irregular outline, fringe-like margin or thread-like outgrowths, color, flat, raised, or umbilicated, etc. On microscopic examination the presence or absence of granules, whether fine or coarse, their arrangement and distribution, should be observed, as well as the finer details of the characteristics mentioned under the naked-eye examination. The observations are usually made with a low-power objective, No. 2 or No. 3. The fine details may be observed with a higher lens, and the arrangement of the bacteria especially at the edge of the colony can be observed. The accompanying figures illustrate the colony characteristics mentioned.

*Determination of the Number of Bacteria.*—If a measured amount of fluid is mixed with a plating medium we should theoretically be able to determine the number of bacteria by counting the number of colonies that develop. This would be true if each bacterium was completely separate, if each was able to multiply, if the medium was satisfactory for the development of each variety contained in the material, and if the oxygen access, moisture, and temperature of incubation were favorable for the growth of all the varieties. This is an impossibility. Most nearly accurate results are obtained where only one or at most several varieties are present and the conditions for their growth maintained. Although the method gives an incorrect result when mixed material is used, the enumeration of the colonies that develop is of a great deal of practical value, as in the examination of milk and water. If the methods employed

are always the same, the comparison of the results gives us valuable information as to the bacterial content.

The method employed is in general as follows: To 9 c.c. of water is added 1 c.c. of the material to be examined; this is thoroughly shaken to separate the organisms; 1 c.c. of this is then added to 9 c.c. of water thoroughly shaken, and 1 c.c. of this added to 9 c.c. of water and so on. This gives a dilution of



FIG. 42.—Irregular fringed colony (*B. malignant edema*). (From Kolle and Wassermann.)



FIG. 43.—Colonies of typhoid and colon bacilli in rather soft gelatin.

1 to 10, 1 to 100, 1 to 1000, etc. How far to dilute the material must be determined from the character and probable bacterial content. One c.c. of each of these dilutions is placed in a Petri dish and the melted plating medium poured into the dish, the two being mixed by tilting the dish. Mixing can be done by adding the dilutions to the tubes of plating medium and pouring the content into the dishes. Though in this way all the medium cannot be poured out of the tube and some bacteria are left behind, it is easier to obtain



FIG. 44.—Colony of colon bacilli grown in soft gelatin.



FIG. 45.—One large irregular colony of colon and two smaller colonies of typhoid bacilli in soft gelatin. (Figs. 43 to 45 from photographs by Dunham.)

even distribution of the colonies in this way, and for comparative work the method gives reliable results. The counts by this method average about 10 per cent. lower than where mixing is done in the dish.

In place of dilutions made by pipettes, a series of loops or rings on a platinum needle can be employed which hold a known quantity (small) of the material to be examined.



To obtain a satisfactory count the colonies should be about 100 to 200 to a plate. Lower numbers than this are too few to give a fair average, higher numbers are difficult to count because the colonies have not sufficient space for



FIG. 46.—Moist raised colonies with no visible structure, looking like a drop of water. (Figs. 46-53 from Lehman and Neumann.)



FIG. 47.—Deep colonies, usually either light brown, gray, or yellow in color, opaque with little marking.

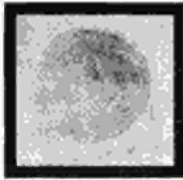


FIG. 48. — The colony very finely granular.



FIG. 49.—Colonies opaque in centre with lighter borders. The margin is coarsely granular, or has twisted threads.

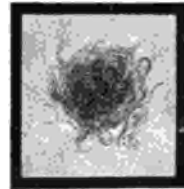


FIG. 50.—Colony in gelatin. The centre is coarsely granular in partly fluid gelatin. The borders are formed of wavy bands of threads.

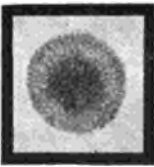


FIG. 51. — Colonies circular in form, composed of radiating threads.



FIG. 52. — Colonies with opaque centres, with a thin border fringe.

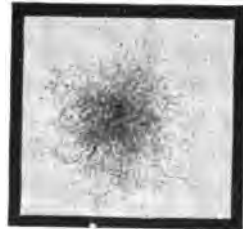


FIG. 53.—Colony showing a network of threads which is thicker in the centre.

full development, some of the colonies become confluent, and some of the feeble bacteria are checked in their development by the crowding and do not develop colonies. The plate that shows about 100 colonies is chosen, therefore, for counting. When no one plate is completely satisfactory in number and even

distribution of the colonies, two of the nearest satisfactory plates are counted and the results averaged. Whenever possible all the colonies on a plate should be counted. Where the colonies are crowded it is necessary to divide the plate into sections to facilitate counting. This is best done by placing the Petri



FIG. 54.—Photograph of a large number of colonies developing in a layer of gelatin contained in a small Petri dish. Some colonies are only pin-point in size; some as large as the end of a pencil. The colonies here appear in their actual size.

dish on a Wolffhügel counting plate, which is a glass plate ruled in square centimeters, some of the squares being still further divided into 9 small squares. A row of squares is counted and then a row at right angles to the first, to obtain a fair average. The number of colonies is divided by the number of squares counted. If the usual 10 c.c. Petri dish is used, the number per square centimeter is multiplied by 63, the number of square centimeters on the dish, to



FIG. 55.—Well-distributed colonies in agar in portion of Petri dish.



FIG. 56.—Wolffhügel's apparatus for counting colonies.

give the total number. This is multiplied by the dilution used in this dish, which gives the colonies developing from 1 c.c. of the material used for plating. If dishes of another size are used, the area in square centimeters is determined by multiplying the diameter by 3.1416.

**Methods of Inoculating Culture Media.**—The inoculation of plating media has been described under the methods of isolating pure cultures. Platinum wire is usually employed for transferring the growth from one medium to another. The wire is either attached to the end of a glass rod or to special aluminum holders which are made for this purpose. Straight wire or wire with the end bent into a loop is used. The wire is sterilized by heating it in the flame of a Bunsen burner until red-hot. Care must be taken that the wire is cool before it is used. The straight wire is used for fishing colonies or



FIG. 57.—Incubator.

where tubed solid media is to be inoculated by long puncture through the media (stab culture). The loop is used for ordinary transfers. In transferring from solid media the loop is drawn upward over the medium, filling the loop with the growth; this is rubbed over the surface of solid media or in the case of fluid media against the glass at upper level of the fluid. In transferring fluid cultures a loop of the fluid is used. When the growth on fluid media is in the form of a pellicle it may be necessary to inoculate this pellicle so that it floats or growth may not take place. In transplanting, the tubes or other containers must not be kept open any longer than necessary or contamination

is likely to occur. In transferring from and to tubed medium the tubes are held by placing them against the palm and fingers of the left hand, grasping the tubes by the butt between the thumb and the palm, low enough so that the contents of the tube are not covered by the thumb. The right hand holding the sterilized platinum wire is used to remove the cotton plugs, one being grasped by the small finger and the other between the fourth and fifth fingers. After drawing out the plugs the necks of the tubes are flamed and the growth then transferred and the plugs replaced; the platinum wire is then sterilized. The plugs should be protected from dust and just before use should be moistened with a weak antiseptic.

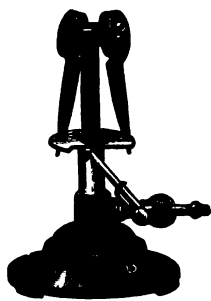


FIG. 58. — Safety-burner.

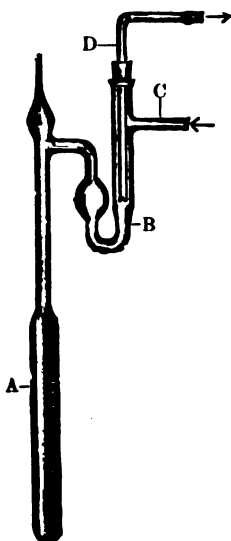


FIG. 59.—Heat regulator. (Dunham.)

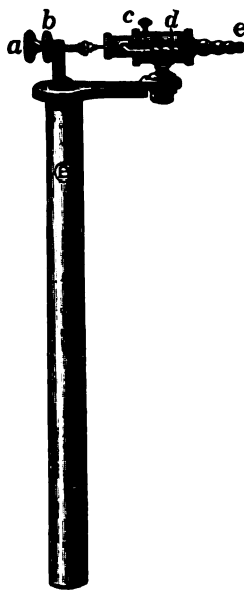


FIG. 60. — Heat regulator. (Roux.) Bimetallic.

**Apparatus for and Methods of Incubation.**—The selection of the temperature for incubation depends on the organism to be grown and the medium employed. Although the optimum temperature for growth may vary between  $5^{\circ}$  and  $50^{\circ}$  C. or more, the great majority of organisms will grow at either  $22^{\circ}$  or  $37^{\circ}$  C. These are the temperatures usually employed. For general purposes the former is obtained by incubation at room temperature. Where a more exact and uniform temperature is necessary an incubator must be employed. This is a double-walled oven, the space between the walls being filled with water; heat is supplied by gas or electricity and cooling by the addition of cold water. The latter is only necessary when the outside temperature rises above  $22^{\circ}$  C. The heating or the addition of water is controlled by an automatic regulator. To maintain a temperature of  $37^{\circ}$  C. a similar incubator is employed, the heating, however, being continuous, an automatic regulator controls the temperature. The heat-controlling devices are of varied construction, the expansion of a fluid or the change in shape of a bar of two unequally expanding metals is used to make and break the electric contact or open and close the gas vent. In the former mercury is used alone or at the point of control. When a large incubating room is required, a room can be heated with a large gas stove, a funnel being placed over the stove and connected with this a radia-

tor-like arrangement of 4- to 6-inch pipes ending in a chimney-like outlet for the products of combustion. The radiator distributes the heat more evenly through the room. In spite of this there will be a variation in temperature in different parts of the room, the shelves near the radiator or nearest the ceiling being the warmer. This is an advantage rather than a disadvantage, as a range of about 5° is available for special purposes. In using gas, all connections are best made of metal; flexible metal tubing is available. Small burners are preferably of the safety type, which, if extinguished, turn off the gas automatically. In the case of gas stoves there should be an independent pilot flame not influenced by the regulator, which will relight the stove in case of accident.

**Oxygen Requirement Methods.**—Where aerobic conditions are required the access of air to the growth is all that is required. For anaerobic conditions the oxygen must be removed. This is done (1) by exhaustion of the air, (2) by chemical absorption of the oxygen, (3) by displacement of the air as by hydrogen, or (4) by growing in the depths of solid media, or in fluid media under a layer of albolene to prevent the reabsorption of air driven off during sterilization; (5) or, lastly, by a combination of these methods. The addition of fresh animal tissues to media for the enhancement of the growth of anaerobic organisms has been mentioned under Media. Occasionally reducing substances are added directly to the medium, or the organism is grown in symbiosis with an aerobic bacillus which absorbs the oxygen.

The group of organisms spoken of as "micro-aërophilic" will only grow when the oxygen is partly removed. This may be done (1) by exhaustion of the air to measured degree, (2) by growing the organism in a sealed jar containing also a stated number of cultures of an organism that absorbs oxygen in its growth, as *B. subtilis*, or (3) by mixing the material with melted agar in tubes and allowing the agar to set, the development taking place at the depth that has the favorable oxygen content. In the second method the optimum number of square centimeters of *B. subtilis* culture per liter of air must be known.

**Anaerobic Methods.**—For exhaustion a pump is required; the filter pumps for attachment to the faucet are the most convenient where water under pressure is available. Any type of pump, however, may be employed. The extent of exhaustion can be measured by a manometer. Any type of jar with a tightly fitting cover and an opening with a stop-cock and of appropriate dimensions for purpose can be used. The Novy jar is a satisfactory type. Museum jars with a side opening are very satisfactory substitutes. The jar must be absolutely tight or the vacuum will not be held. Absorption can be added to exhaustion by placing pyrogalllic acid and a stick of sodium hydrate in the bottom of the jar. After exhaustion is completed a small amount of water is allowed to be sucked back into the jar. This dissolves the sodium hydrate and the resulting reaction of the sodium hydrate with the pyrogalllic acid is accompanied by absorption of the oxygen from the air remaining in the jar.

Hydrogen may be used to replace the exhausted air. After exhaustion, hydrogen from a Kipp apparatus is sucked back into the jar diluting the remaining air; the jar is again exhausted and then filled with hydrogen, again exhausted and filled. Only traces of air will remain, and if absolute anaërobiasis is required pyrogalllic acid may be used to absorb this last trace as above, allowing the water to be sucked back before the negative pressure is completely relieved by the hydrogen. In relieving the negative pressure it may be necessary to use a pneumatic trough to collect the hydrogen, as the generation of hydrogen will be too slow and air will be drawn through the Kipp apparatus. It is advisable not to relieve the negative pressure completely, as this helps to hold the lid tightly in place, and furthermore, the subsequent expansion when the jar is incubated will tend to loosen the lid and allow inward diffusion of air. When the pyrogalllic method is used, the tubes or plates must be raised above the level of the fluid. Of the methods given, the second and third are the most satisfactory. The absorption method may be used alone, either using a jar to hold the tubes or plates, or individual plates or tubes may be handled as in the following:

*Buchner's Method.*—The culture tube is placed in a larger tube, at the bottom of which is placed the pyrogalllic acid, on which some sodium hydrate is poured and the tube quickly closed with a rubber stopper, or the NaOH may be added dry and water added.

*Wright's Method.*—The tubes are plugged with absorbent cotton; after inoculation of the medium the plug is pushed into the tube, pyrogalllic placed on the stopper, sodium hydrate solution added, and the tube quickly corked.

*Zinsser's Method for Plates.*—The dishes employed must be deeper than the ordinary Petri dishes. The agar is poured in the smaller dish in the ordinary manner, and as soon as hard inverted over moistened filter paper to protect it from contamination. In the larger dish is placed the pyrogalllic acid and the smaller dish placed in it. One side of the smaller dish is raised and the hydrate solution poured in the space between the two dishes and the inner dish dropped back. As rapidly as possible albolene is run in the space between the dishes from a pipette previously filled so that no time is lost.

*The methods of exclusion of oxygen* are simple and can be, in most instances, substituted for the more cumbersome methods described. Plating may be done with the ordinary Petri dishes as follows (Krumwiede and Pratt): The bottom part of the dish, open side up, is placed in the cover and sterilized in this shape and protected from dust until used. The plating medium is inoculated as though pour plates were to be made in the ordinary way. The



FIG. 61.—Novy jar for anaerobic cultures



FIG. 62.—Buchner's anaerobic tube  
The fluid consists of pyrogalllic acid dissolved in 10 per cent. NaOH solution. By Wilson's method the tubes are charged with pieces of caustic potash covered with pyrogalllic acid.

inoculated medium is then poured into the cover and the bottom of the dish laid on the fluid medium, tilting it so that the air can escape as it falls into place. With a little practice this can be done so that no bubbles are present. After the medium has set the edge is painted over with melted paraffin to

prevent drying and contamination. If any of the medium flows over the edge, it is wiped away by carbolized cotton (see Fig. 63). After incubation the parts of the dish are carefully pulled apart and the colonies may be fished. As a rule the agar remains in the cover. If it should adhere to the other part of the dish, this can be laid in the cover to allow of fishing under the microscope. If gas-producing bacteria are present, fishing should be done as soon as sufficient growth has taken place or the gas will disrupt the medium and cause the growth to spread over the plate. If an actively motile organism is present, the method may fail because of spreading between the medium and the glass.

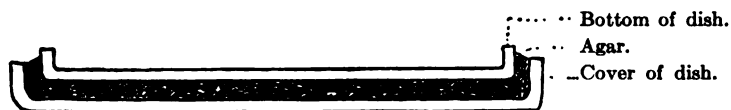


FIG. 63.—Anaerobic plating method.

Another method for the isolation of pure cultures is the *shake culture*. The material is diluted with a plating medium in the usual way, although more medium is used in the tubes. Instead of pouring the medium into plates the medium is allowed to solidify in the tubes. The colony desired can be obtained by scratching the tube with a file at the level of the colony, then wet the tube with alcohol, burn this off and press on the scratch with a very hot piece of metal or glass rod. The tube will crack and the column of agar can be cut through to allow the separation of the two parts of the tube and the colony fished. This method has a limited application, but is especially useful when the organism to be isolated is a partial anaërobie and grows at a certain level in the agar. Pure cultures of these organisms may not be obtained at the first attempt, but the level of thickest growth may then be used, the first culture serving in this way as an enrichment.

For the cultivation of pure cultures the use of stab cultures in agar or gelatin may be used. The most satisfactory medium for this purpose is a semisolid agar (Krumwiede and Pratt). Because of the softness of the medium the material for subinoculation is easily obtained; the stab inoculation closes better than if made in stiffer medium, and the medium does not dry out nor split so readily. For subinoculation a platinum wire bent like a corkscrew is a help in obtaining the material from the depths of the media. With spirochetes it is much easier to obtain the growth with the capillary pipette from about the tissue at the bottom of the tube than where the usual 1.5 per cent. is employed. The use of the capillary pipette for the transfer of cultures is open to much wider application with semisolid media, because if the medium has a satisfactory density, the ordinary rubber teat exerts sufficient suction to draw up the medium and a syringe is not needed.

The *exclusion of oxygen* for fluid medium is most simply carried out by sterilizing the medium under a layer of albolene. This can be done either in tubes or flasks, preferably the globe flasks. In the latter the medium is filled to the neck and albolene added, leaving room for expansion; after sterilization and contraction of the medium more sterile albolene is added if necessary.

Occasionally anaërobes are grown in mixed culture with an aerobic organism to absorb the oxygen. One of the best organisms for this purpose is the hay bacillus.

As a general rule the addition of a fermentable carbohydrate to the medium gives much better growth of anaerobic organisms.

**The Study of Pure Cultures.**—The study of pure cultures is the basis for classification and identification. The characteristics studied are: the morphology, staining reactions, and motility when grown on or in

both solid and fluid media (see Microscopic Study); the cultural characteristics when grown on various media, its food, oxygen, and temperature requirements, its reaction to the various immunity tests (see chapters on Immunity), and the action of the organism or its products on experimental animals (see Use of Animals). The following is a short summary of the more important cultural reactions and the methods of eliciting them.

*Cultural Characteristics.—Colony Morphology.*—(See Application of Plating Methods.)

*Growth on Agar and Other Solid Media (Surface Growth).*—Moist or dry, flat or raised, flaky or easily emulsified, adherent to medium or not, mucoid or slimy, smooth, irregular, or threaded margin, color or pigment production (see below), extension into medium, etc.

*Growth in Solid Media.*—In stab culture, depth to which growth extends, character of growth around the surface puncture, the spreading of the growth into the medium (an index of motility best shown in semisolid media), thread-like extensions from the line of puncture, in gelatin, liquefaction, non-liquefaction, rate, area and character if present. In shake cultures, the character of the colonies.

*Fluid Media.*—Pellicle, tenacious or easily broken, spreading up the side of the tube or not, thick or thin, clear or cloudy, and degree of clouding, sediment or deposit on sides of tube, granular, flaky, gelatinous, mucoid, or stringy, stalactite formation, more densely clouded at one level than another.



FIG. 64.—Stab cultures of three cholera spirilla in gelatin, showing in upper portion of growth considerable liquefaction of nutrient gelatin.

*Food Requirements.*—Growth on simple or complex media, albumins such as serum required, blood or hemoglobin necessary, carbohydrate needed for full development (example glycerin for tubercle bacillus).

*Oxygen Requirements.*—Growth only in free oxygen, obligate aërobe; growth only where oxygen is absent (depths of media, etc.), obligate anaërobe; growth under both conditions, facultative anaërobe (an available carbohydrate is necessary to elicit the facultative character); growth with a definite but lessened amount of oxygen (development only in one level of solid media), micro-aërophilic. This last characteristic may be lost after cultivation for some time on artificial culture media.

*Optimum Temperature.*—Not often used, but may furnish absolute evidence of the dissimilarity of two organisms. Not only the optimum but also the range, especially lower, should be determined.



**Pigment Production.**—The color and shade, the optimum medium and temperature for its production, limited to the area of growth, or diffused through the medium; its solubility in extracting agents, as alcohol, ether, chloroform, etc.; more than one pigment present, as shown by use of different extractives. In the case of the *coercææ* especially, the presence of pigment and its color and shade may be determined with ordinary agar; the growth is taken up with a platinum loop and spread on a piece of white glazed paper. Although the presence of pigment may be completely obscured on the agar, its presence is immediately evident on the paper.

**Ferment Action** (Cultural Evidence of).—The most important culturally are those causing fermentation of carbohydrates. Less important are proteolytic, diastatic, inverting, and rennin-like ferments.

**Fermentation of Carbohydrates.**—The sugars selected will depend on the organism to be identified as well as the medium selected as a basis for the addition of the sugar. The preparation and the use of indicators or titration to determine the production of acid and the use of fermentation tubes to demonstrate gas production have been described. The amount of gas is expressed in terms of percentage, thus if the closed arm is half-full of gas, 50 per cent., etc. To determine roughly proportion of carbon dioxide and of hydrogen, mark on tube quantity of gas produced, then fill the bulb with sodium hydroxide solution (10 per cent.) and insert a rubber stopper; the tube is inverted several times to mix and the gas again collected in the closed arm. The gas absorbed is carbon dioxide; the remainder is usually hydrogen. In the case of media containing coagulable proteins, as in milk, serum water, serum broth, acid production, if sufficient, is followed by coagulation; the coagulation is shown in solid media by precipitation or opacity. The ability to attack a carbohydrate may differ under aerobic and anaerobic conditions.

**Proteolytic action** is shown by gelatin liquefaction, liquefaction of Löffler's blood serum, digestion of milk. Prolonged incubation even for a month may be needed. The production of peptone may also be used for determining the digestion of albuminous media. Note odor and, if present, its character.

**Alkali Production.**—Determined by indicators or titration; note in milk cultures especially. An initial acidity may be noted, due to traces of a fermentable carbohydrate. The cultures may have to be incubated for one to three weeks. Milk on prolonged incubation may become translucent whether due to alkali production or to a proteolytic enzyme not determined.

**Sulphuretted hydrogen**, to demonstrate, use peptone water cultures to which is added 1 per cent. of a 1 per cent. solution of lead acetate or ferric tartrate; the precipitate which forms on mixing these turns black if sulphuretted hydrogen is produced.

**Nitrate Production.**—Grow in the following medium. Two solutions are necessary for the test.

1. Naphthylamin . . . . .	0.1 gram
Distilled water . . . . .	20.0 c.c.
Acetic acid 25 per cent. . . . .	150.0 c.c.

Dissolve the naphthylamin in the water by means of heat, cool, filter, and add the acetic acid.

2. Sulphanilic acid . . . . .	0.5 gram.
Acetic acid dil. 1 to 16 . . . . .	150.0 c.c.

Keep them separate and mix in equal parts as needed.

To about 4 c.c. of the culture add 2 c.c. of the mixed solutions. The development of a pink color shows the presence of nitrates, the intensity of the color being proportional to amount present.

Similar reduction processes are seen in the decolorization of litmus and some of the aniline dyes. The bacteria utilize the oxygen of the dyes, reducing them to their leuco-bases.

## DETAILED FEATURES.

NOTE—Underscore required terms. Observe notes and glossary of terms on opposite side of card.

## I. MORPHOLOGY (?)

1. Vegetative Cells, Medium used . . . . . temp. . . . . age . . . . . days  
Form, round, short rods, long rods, short chains, long chains, filaments, commas, short spirals, long spirals, clostridium, cuneate, clavate, curved.  
Limits of Size . . . . .  
Size of Majority . . . . .  
Ends, rounded, truncate, concave.  
Agar { Orientation (grouping) . . . . .  
Hanging-block { Chains (No. of elements) . . . . .  
Short chains, long chains  
Orientation of chains, parallel, irregular.
2. Sporangia, medium used . . . . . temp. . . . .  
age . . . . . days  
Form, elliptical, short rods, spindled, clavate, drumsticks.  
Limits of Size . . . . . Size of Majority . . . . .  
Agar { Orientation (grouping) . . . . .  
Hanging-block { Chains (No. of elements) . . . . .  
Orientation of chains, parallel, irregular.  
Location of Endospores, central, polar.
3. Endospores.  
Form, round, elliptical, elongated.  
Limits of Size . . . . .  
Size of Majority . . . . .  
Wall, thick, thin.  
Sporangium wall, adherent, not adherent.  
Germination, equatorial, oblique, polar, bipolar, by stretching.
4. Flagella, No. . . . . Attachment polar, bipolar, peritrichate. How Stained . . . . .
5. Capsules, present on . . . . .
6. Zoogles, Pseudozoogles . . . . .
7. Involution Forms, on . . . . . in . . . . . days at . . . . ° C.
8. Staining Reactions.  
1 : 10 watery fuchsin, gentian violet, carbol-fuchsin, Loeffler's alkaline methylene blue.  
Special Stains,  
Gram . . . . . Glycogen . . . . .  
Fat . . . . . Acid-fast . . . . .  
Neisser . . . . .

## II. CULTURAL FEATURES (?)

1. Agar Stroke.  
Growth, invisible, scanty, moderate, abundant.  
Form of growth, filiform, echinulate, beaded, spreading, plumose, arborescent, rhizoid.  
Elevation of growth, flat, effuse, raised, convex.  
Lustre, glistening, dull, cretaceous.  
Topography, smooth, contoured, rugose, verrucose.  
Optical characters, opaque, translucent, opalescent, iridescent.  
Chromogenesis (?) . . . . .  
Odor, absent, decided, resembling . . . . .  
Consistency, slimy, butyrous, viscid, membranous, coriaceous, brittle.  
Medium, grayed, browned, reddened, blued, greened.
2. Potato.  
Growth scanty, moderate, abundant, transient, persistent.  
Form of growth, filiform, echinulate, beaded, spreading, plumose, arborescent, rhizoid.  
Elevation of growth, flat, effuse, raised, convex.  
Lustre, glistening, dull, cretaceous.  
Topography, smooth, contoured, rugose, verrucose.  
Chromogenesis (?) . . . . . Pigment in water insoluble, soluble: other solvents . . . . .  
Odor, absent, decided, resembling . . . . .  
Consistency, slimy, butyrous, viscid, membranous, coriaceous, brittle.  
Medium, grayed, browned, reddened, blued, greened.
3. Loeffler's Blood-serum.  
Stroke invisible, scanty, moderate, abundant.  
Form of growth, filiform, echinulate, beaded, spreading, plumose, arborescent, rhizoid.  
Elevation of growth, flat, effuse, raised, convex.  
Lustre, glistening, dull, cretaceous.  
Topography, smooth, contoured, rugose, verrucose.  
Chromogenesis (?) . . . . .  
Medium grayed, browned, reddened, blued, greened.  
Liquefaction begins in . . . . . d, complete in . . . . . d.
1. Agar Stab.  
Growth uniform, best at top, best at bottom: surface growth scanty, abundant: restricted, wide-spread.  
Line of puncture, filiform, beaded, papillate, villous, plumose, arborescent: liquefaction.

5. Gelatin Stab.  
Growth uniform, best at top, best at bottom  
Line of puncture, filiform, beaded, papillate, plumose, arborescent.  
Liquefaction crateriform, napiform, infusaccate, stratiform: begins in . . . . . d  
complete in . . . . .  
Medium fluorescent, browned.
6. Nutrient Broth.  
Surface growth, ring, pellicle, flocculent, none.  
Clouding slight, moderate, strong: transient: none: fluid turbid.  
Odor, absent, decided, resembling . . . . .  
Sediment, compact, flocculent, granular, on agitation, abundant, scant.
7. Milk.  
Clearing without coagulation.  
Coagulation prompt, delayed, absent.  
Extrusion of whey begins in . . . . . d  
Coagulum slowly peptonized, rapidly peptonized  
Peptonisation begins on . . . . d, complete on Reaction, 1d. . . . 2d. . . . 4d. . . . 10d. . . .  
Consistency, slimy, viscid, unchanged.  
Medium browned, reddened, blued, greened.  
Lab ferment, present, absent.
8. Litmus Milk.  
Acid, alkaline, acid then alkaline, no change. Prompt reduction, no reduction, partial slow.
9. Gelatin Colonies.  
Growth slow, rapid.  
Form, punctiform, round, irregular, amoeboid, filamentous, rhizoid.  
Elevation, flat, effuse, raised, convex, pulvinate (liquefying).  
Edge, entire, undulate, lobate, erose, lacerate, filamentous, floccose, curled.  
Liquefaction, cup, saucer, spreading.
10. Agar Colonies.  
Growth slow, rapid (temperature . . . . .  
Form, punctiform, round, irregular, amoeboid, filamentous, rhizoid.  
Surface smooth, rough, concentrically ring striate.  
Elevation, flat, effuse, raised, convex, pulvinate.  
Edge, entire, undulate, lobate, erose, lacerate, floccose, curled.  
Internal structure, amorphous, finely, coarse, grumose, filamentous, floccose, curled.
11. Starch Jelly.  
Growth, scanty, copious.  
Diastatic action, absent, feeble, profound.  
Medium stained . . . . .
12. Silicate Jelly (Fermi's Solution).  
Growth copious, scanty, absent.  
Medium stained . . . . .
13. Cohn's Solution.  
Growth copious, scanty, absent.  
Medium fluorescent, non-fluorescent.
14. Uchinsky's Solution.  
Growth copious, scanty, absent.  
Fluid viscid, not viscid.
15. Sodium Chloride in Bouillon.  
Per cent. inhibiting growth . . . . .
16. Growth in Bouillon over Chloroform, u feeble, absent.
17. Nitrogen. Obtained from peptone, aspartic acid, urea, ammonia salts, nitrogen.
18. Best media for long-continued growth . . . . .
19. Quick tests for differential purposes . . . . .

## III. PHYSICAL AND BIOCHEMICAL FE

	Dextrose	Saccharose	Lactose	Maltose	Glycerin	Starch
1. Fermentation-tubes containing peptone-water or sugar-free bouillon and						
Gas production, in per cent.						
(H) (CO <sub>2</sub> )						
Growth in closed arm						
Amount of acid produced 1d.						
Amount of acid produced 2d.						
Amount of acid produced 3d.						

[illegible]

1. **Pathogenic to Animals.**  
*Insects, crustaceans, fishes, reptiles, birds, mice, rats, quinea-pigs, rabbits, dogs, cats, sheep, goats, cattle, horses, monkeys, man*.....
2. **Pathogenic to Plants:**  
.....  
.....  
.....  
.....
3. **Toxins, soluble, endotoxins.**
4. **Non-toxin forming.**
5. **Immunity bactericidal.**
6. **Immunity non-bactericidal.**
7. **Loss of virulence on culture-media:** prompt, gradual,  
not observed in..... months.

MORPHOLOGY (°)	Diameter over 1µ		
	Chains, filaments		
	Endospores		
	Capsules		
	Zoogleea, Pseudozoogleea		
	Motile		
	Involution forms		
	Gram's stain		
	CULTURAL FEATURES (°)	Broth	Cloudy, turbid
			Ring
Pellicle			
Sediment			
Agar		Shining	
		Dull	
		Wrinkled	
		Chromogenic	
Gel. plate		Round	
		Proteus-like	
		Rhizoid	
		Filamentous	
Gel. Slab.		Curled	
		Surface growth	
Potato		Needle growth	
		Moderate, absent	
		Abundant	
		Discolored	
BIOCHEMICAL FEATURES		Liquefaction	Starch destroyed
			Grows at 37° C.
	Grows in Cohn's sol.		
	Grows in Uchinsky's sol.		
	Milk	Gelatin (°)	
		Blood-serum	
		Casein	
		Acid curd	
	DISTRIBUTION	Rennet curd	
		Casein peptonized	
Indol (°)			
Hydrogen sulphide			
Ammonia (°)			
Nitrates reduced (°)			
Fluorescent			
Luminous			
DISTRIBUTION	Animal pathogen, epizoon		
	Plant pathogen, epiphyte		
	Soil		
	Milk		
	Fresh water		
	Salt water		
	Sewage		
	Iron bacterium		
Sulphur bacterium			

*Aromatic Products (Indol Production).*—The usual medium is peptone water. Two methods are available for testing: (1) Salkowski Method. To the culture add several drops of concentrated sulphuric acid or 1 c.c. of a 10 per cent. solution and then add 1 c.c. of a 1 to 10,000 sodium sulphite solution. A pink color develops at the point of contact with the acid, which on shaking diffuses throughout. (2) Ehrlich Method. This test is more constant and reliable. To the culture add 1 c.c. of a 2 per cent. solution of paradimethylaminobenzaldehyde in 95 per cent. alcohol and then add, drop by drop, concentrated hydrochloric acid until a red zone appears at the point of contact of the alcohol and the peptone. Not more than 0.5 c.c. of the acid is required. On standing the zone deepens and widens. The color is soluble and the test should be confirmed by shaking with chloroform, which dissolves out the color. The tests for indol are made after four to six days' incubation. Kligler gives a good resumé of the subject.

*Cholera-red Reaction.*—Nitrate is produced by some bacteria from the nitrate present as an impurity in the peptone, and the red color appears on the addition of acid alone.

Both of the above tests are interfered with by the presence of a fermentable carbohydrate.

*Voges-Proskauer Reaction.*—Grow for three days in 2 per cent. glucose-peptone solution in fermentation tubes. Add 2 or 3 c.c. of a strong solution of potassium hydrate. A pink color develops on exposure to the air.

*Toxin Production.*—The presence of a toxin is not usually elicited by cultural methods; one exception is the demonstration of hemolysins by plating with blood agar. The hemolysis is shown by a clearing about the colony.

For other methods of demonstrating the production of toxin, and the use of immunological reactions and animal tests see the appropriate sections of this work.

The accompanying chart is given as an example of a complete cultural study of an organism and the methods of using cultural reaction for classification. The characteristics to be elicited will depend on the group of organism studied. In the case of many of the organisms pathogenic for man and animal, the cultural characteristics only give us a presumptive identification, the final identification depending on animal and immunological tests.

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## CHAPTER V.

### THE USE OF ANIMALS FOR DIAGNOSTIC AND TEST PURPOSES.

SUITABLE animals are necessarily employed for many microbiological purposes. (1) To obtain a growth of varieties that for any reason grow with difficulty on artificial culture media, as in the case of tubercle bacilli, material suspected of containing the bacilli is injected into guinea-pigs, with the knowledge that, if present, although in too small numbers to be detected by microscopic or culture methods, the bacilli will develop in the animals' bodies, and thus reveal themselves. The same may be true of glanders, tetanus, and anthrax bacilli, of pneumococci, of other bacteria, and of protozoa. Certain micro-organisms have not yet been grown on artificial media. This is true of few bacteria, of most protozoa, of many of the spirochetes, and of certain unknown infectious agents such as produce smallpox and Rocky Mountain spotted fever. (2) To cause an increase of one variety of organisms in a mixture and thus obtain a pure culture. An injection of sputum subcutaneously in rabbits may give rise to a pure pneumococcus septicemia or a pure tuberculosis. (3) To test virulence: Animals are used to test the virulence or toxin production of organisms, where, as in the case of diphtheria, we have very virulent, attenuated, and non-virulent bacilli of, so far as we know, identical cultural characteristics. Here the injection of a susceptible animal, such as the guinea-pig, is the only way that we can differentiate between those capable of producing diseases and those that are harmless. Still another use of the animals is to differentiate between two virulent organisms, which, though entirely different in their specific disease poisons, are yet so closely allied morphologically and in culture characteristics that they cannot always be separated except by studying their action in the animal body both with and without the influence of specific serums. In this way the typhoid and colon bacilli may be separated, or the pneumococcus and streptococcus. (4) To test the antitoxic or microbicidal strength of sera: Diphtheria antitoxin is added to diphtheria toxin and injected into guinea-pigs, and streptococcus immunizing serum is mixed with living streptococci and injected into the vein of a rabbit. (5) To produce antitoxic, bactericidal, or agglutinating sera for therapeutic and diagnostic use.

**The Inoculation of Animals.**—The inoculation of animals may be made either through natural channels or through artificial ones:

1. Cutaneous. The material is rubbed into the abraded skin.
2. Intracutaneous. The material is injected into the skin. Important specific local reactions may be obtained by this method.

3. Subcutaneous. The substances are injected by means of a hypodermic needle under the skin, or are introduced by a platinum loop into a pocket made by an incision.

4. Intravenous. The substances are injected by means of a hypodermic needle into the vein. This is usually carried out in the ear vein of the rabbit. If rabbits are placed in a holder, so that the animal remains quiet and only the head projects, it is usually easy to pass a small needle directly into one of the ear veins, especially those running along the edge of the ear. If the ear is first moistened with a 3 per cent. carbolic acid solution, and then supported between the finger inside and the thumb outside, the vein is usually clearly seen and entered with ease, if a small, sharp needle is held almost parallel with the ear surface and gently pushed into it. When no holder is obtainable, the rabbit can be held by an assistant seizing the forelegs in one hand and the hind in another and holding the rabbit head downward, or the animal may be held between the knees of the operator, its body resting on the operator's apron.

5. Into the anterior chamber of the eye.

6. Into the body cavities. The peritoneal and less often the pleural cavities are used for microbial injection. The hypodermic needle is usually employed, less often a glass tube drawn out to a fine point. The needle or the pointed glass tube is gently pushed through the abdominal wall, moved about to be certain that the intestines have not been perforated and the fluid injected.

7. By inhalation. This method is carried out by forcing the animal to inhale an infected spray or dust.

8. By the trachea. This method is carried out by making an incision in the trachea and then inoculating the mucous membrane or injecting substances into the trachea and bronchi.

9. Through the intestinal tract by swallowing or by the passage of a rubber tube. Morphin may be given to prevent peristalsis.

10. Into the brain substance or ventricles after trephining, or when the parietal bones are thin, as in the guinea-pig and the rabbit, after making a tiny opening with the point of a small, heavy scalpel.

Mice, which are usually inoculated subcutaneously in the body or at the root of the tail, are best placed in a mouse holder, but can be inoculated by grasping the tail in a pair of forceps, and then, while allowing the mouse to hang head downward in a jar, a glass plate is pushed across the top until only space for its tail is left.

Monkeys and apes are used for certain infections, such as syphilis and smallpox, where only they and man are markedly susceptible.

All these methods must be carried out with the greatest care as to cleanliness, the hair being clipped and the skin partially, at least, disinfected. The operator must be careful not to infect himself or his surroundings. After the inoculations the animals should be given the required food and kept in appropriate quarters. For food, rabbits and guinea-pigs usually require only carrots and hay.

When possible, all animals should be anesthetized during painful experiments.

**Autopsies.**—Autopsies should be made at the earliest moment possible, for soon after death some of the species of the bacteria in the intestines are able to penetrate through the intestinal walls and infect the body tissues. If delay is unavoidable, the animals should be put immediately in a place where the temperature is near the freezing-point. In making cultures from the dead bodies the greatest care should be taken to avoid contamination. The skin should be disinfected, and any dust prevented by wetting with a 5 per cent. solution of carbolic acid. All instruments are sterilized by boiling in 3 per cent. washing-soda solution for five minutes. Changes of knives, scissors, and forceps should be made as frequently as the old ones become infected. When organs are examined, the portion of the surface through which an incision is to be made must be sterilized, if there is danger that the surrounding cavity is infected, by searing with the flat blade of an iron spatula which has been heated to a dull red heat. Tissues if removed should be immediately placed under cover so as not to become contaminated. Sterile deep Petri plates are useful for this purpose.

When it is necessary to transport tissues from a distance, they should be wrapped in sterile cloths and sent to the point of destination as soon as possible. In warm weather they may be kept cool by surrounding the vessels which contain them with ice.

Animals rarely show the same gross lesions as man when both suffer from the same infection. The cell changes, however, are similar, and, also, so far as we can test them, the curative or immunizing effects of protective serums.

Animals comparatively frequently undergo spontaneous infections with organisms similar to those producing disease in man. This is particularly so with the streptococcus-pneumococcus group, so one must be constantly on one's guard in working with such organisms.

**Leukocytes for Testing Phagocytosis.**—Inoculate into the pleural cavity of a rabbit 5 c.c. of a thick suspension of aleuronat powder in a boiled starch solution. The solution should be thick enough to hold the aleuronat in suspension. A 20 to 25 per cent. solution of peptone gives good results. The fluid is withdrawn eighteen to twenty-four hours after the injection.

For purposes of obtaining the opsonic index the whole blood is taken. For description of the method see chapter on Opsonins.

Leukocytes from the horse can be readily obtained by mixing the blood with 1 per cent. of sodium citrate and allowing the mixture to stand. The red cells rapidly sink and leave the leukocytes in the supernatant fluid.

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## CHAPTER VI.

### THE PROCURING AND HANDLING OF MATERIAL FOR MICROBIOLOGICAL EXAMINATION FROM THOSE SUFFERING FROM DISEASE.

A LONG experience has taught us that physicians very frequently take a great amount of trouble, and yet, on account of not carrying out certain simple but necessary precautions, make worthless cultures or send material almost useless for microbiological study.

In making cultures from diseased tissues various procedures may be carried out, according to the facilities which the physician has and the kind of information that he desires to obtain. From the dead body culture material should be removed at the first moment possible after death. Every hour's delay makes the results less reliable. From both dead and living tissues, the less the alteration that occurs in any substance between its removal from the body and its examination and inoculation upon or in culture media or animals, the more exact will be the information obtained. If the material is allowed to dry, many microbes will be destroyed in the process, and certain forms which were present will be obliterated or, at least, entirely altered in the proportion which they bear to others. If possible, therefore, smears should be made and culture media should be inoculated directly from the patient or dead body. For the latter purpose a microbiologist should take the most suitable of the culture media to the bedside or autopsy table. Such a list of media, if fairly complete, would comprise nutrient bouillon alone and mixed with one-third its quantity of ascitic fluid, slanted nutrient agar, slanted agar streaked with rabbit or human blood, firmly solidified slanted blood serum and slanted ascitic glucose agar. Additional media will be necessary for special purposes, such as the isolation of typhoid or tetanus bacilli. If only one variety of media is to be used, the solidified blood serum is most useful for parasitic bacteria, and this can be easily carried by the physician and inoculated by him, even if he is not very familiar with bacteriological technic. In the first place some of the infected material should always be smeared on a couple of clean slides or cover-glasses and fixed moist in methyl alcohol or allowed to dry in the air. These can be stained and examined later, and may give much valuable information. For special media in isolation see chapter on Media.

The material must be obtained in different ways, according to the nature of the infection.

For the detection of the bacteria causing septicemia we are met

with the difficulty that there are apt to be very few organisms present in the blood until shortly before death. It will therefore be almost useless to take only a drop of blood for cultures, as even when present there may not be more than eight to ten organisms in a cubic centimeter. If cultures are to be made at all, it is therefore best to make them correctly by taking from 5 to 20 c.c. of blood by means of a sterile hypodermic needle or a suitable glass tube armed with a hypodermic needle, from the vein of the arm after disinfecting the skin with tincture of iodine. To each of five different tubes containing bouillon we add 1 c.c. of blood, and to a flask containing 100 c.c. we add 5 c.c. We have made by this mixture of blood and bouillon a most suitable medium for the growth of all bacteria which produce septicemia, and at the same time have added a sufficient quantity of blood to insure us the best possible chance of having added some of the bacteria producing the disease. We also add to each of several tubes of melted nutrient agar, at 40° C., 1 c.c. of blood and pour the mixture into Petri plates, so as to indicate roughly the number of organisms present by the number of colonies developing. When blood must be carried to a distance, clotting should be prevented by having in the test-tube sufficient 10 per cent. solution of sodium citrate, bile, or ammonium oxalate to prevent clotting.

From wounds, abscesses, cellulitis, etc., the substance for microbiological examination can, as a rule, best be obtained by means of a syringe, or when the lesion is opened, by small rods armed with a little absorbent cotton. A number of these swabs can be sterilized in a test-tube and so carried. The swab is inserted in the wound, then streaked gently over the oblique surface of the nutrient agar in one tube, over the blood serum in another, and then inserted in the bouillon. Finally, either at the bedside or in the laboratory, material is thinly streaked over the surface of nutrient agar contained in several Petri dishes. We inoculate several varieties of media, with the hope that one at least will prove a suitable soil for the growth of the organisms present. From surface infections of mucous membranes, as in the nose, throat, vagina, etc., the swab, again, is probably the most useful instrument for obtaining the material for examination. The greatest care, of course, must be used in all cases to remove the material for study without contaminating it in any way by other material which does not belong to it. Thus, for instance, if we wish to obtain material from an abscess of the liver, where the organ lies in a peritoneal cavity infected with microbes, one must first absolutely sterilize the surface of the liver by pressing on it the blade of a hot iron spatula before cutting into the abscess, so that we may not attribute the infection which caused the abscess to the germs which we obtained from the infected surface of the liver. From such an organ as the uterus it is only with the greatest care that we can avoid outside contamination, and only an expert microbiologist familiar with such material will be able to eliminate the vaginal from the uterine microbes.

A statement of the conditions under which materials are obtained should always accompany them when sent to the laboratory for examination, even if the examination is to be made by the one who made cultures. These facts should be noted, or otherwise at some future date they may be forgotten and misleading information sent out. The work of obtaining material for examination without contamination is at times one of extreme difficulty. It simply must be remembered that if contamination does take place our results may become entirely vitiated, and if the difficulties are so great that we cannot avoid it, it may simply mean that under such conditions no suitable examination can be made. Where the substance to be studied cannot be immediately subjected to cultures or animal inoculations, it should be transferred in a sterile bottle as soon as possible to a location where the cultures can be made. If for any reason delay must take place, the material should at least be put in a refrigerator where cold will both prevent any further growth of some varieties of microbes and lessen the danger of the death of others.

In obtaining samples of fluid, such as urine, feces, etc., the bottles in which they are placed should always be sterile, and, of course, no antiseptic should be added. It is necessary clearly to explain this to the nurse, for she has probably been instructed to add disinfectants to all discharges. Disinfected material is, of course, entirely useless for complete microbiological investigations. It cannot be too much emphasized that materials which are not immediately used should be sent to the laboratory as quickly as possible, for in such substances as feces, where enormous numbers of various kinds of microbes are present, those which we seek most, such as the typhoid bacilli, frequently succumb to the deleterious products of the other microbes present. Even when abundantly present, living typhoid bacilli may entirely disappear from the feces in the course of twelve hours, while at other times they may remain for weeks. These differences depend on the associated organisms present, the chemical constitution of the feces or urine, and the conditions under which the material is obtained. Water and milk rapidly change in their bacterial content if not kept under 40° F.

For obtaining fluid for agglutination and other purposes, blister fluid is valuable. A blister can be raised quickly by placing a piece of blotting paper moistened with a little strong ammonia on the skin and covering with a watch-glass, or one may be more slowly formed by a cantharides plaster.

**Routine Technic Carried Out at Laboratory when Thorough Examination Required.**—As has just been indicated, the microbiological examination proceeds somewhat differently, according to the information needed. When, as is the case with most clinical material, definite knowledge in regard to the presence or absence of a particular organism is desired, the culture media are used which are known to be most suitable for the organisms sought, such as Löffler's blood medium for the

diphtheria bacillus, Petroff medium for the tubercle bacillus, and so on. These media have been already given in the chapter on Culture Media. When, as is generally the case with autopsy material and sometimes with clinical, a complete examination is needed in order to determine unknown organisms, the procedure may be as follows:

1. At the autopsy table the routine cultures and smears are made as described above.

2. Material from the different parts is secured under aseptic precautions in sterile receptacles and taken to the bacteriological laboratory. The receptacles should be surrounded by ice if the laboratory is at a distance.

3. A smear from each part is stained and examined in order to determine in some measure the kind and number of microbes present, so we may more wisely select suitable culture media if other than those already used are needed, and may make the right culture dilutions if these are necessary. Exceptionally, cultures are made before smears are taken.

**Use of Gram's Stain.**—Gram's stain gives more information as to the kind of germs present than any other one stain, so when possible this stain should be used. Other stains, however, may help, if for any reason Gram's is not at hand; and smears made from blood or from suspected syphilitic material should be stained by Giemsa's method or an equivalent (see Staining Methods for formulas of stains). Of course, if one is looking for a special organism, the special stain for that organism should be used.

A Gram-stained smear may show all Gram-negative or all Gram-positive microbes or a mixture of the two, or it may show a number only partially stained (Gram-amphiphile).

The following points must be remembered in using the stain and in interpreting the results:

- (a) The smears should be thin and evenly spread.
- (b) The staining solutions should be fresh (aniline-water-gentian violet lasts about three weeks).
- (c) Controls, fresh cultures (about twenty-four hours old) of a Gram-negative and a Gram-positive bacterium, should be used on the same slide with the smear to be examined.
- (d) If there is much albumin in the suspected material less heat should be used in fixing.
- (e) If the urine is very acid, the results may not be good.
- (f) Mix urinary sediment with egg albumen, better to fix it, and wash out urinary salts with tap-water and stain.
- (g) Too much dependence should not be placed upon the finding of Gram-negative microbes in tissues, since organisms which in pure young cultures may be positive to Gram, show forms as they grow older both in tissues and in cultures, intermediate between the positive and negative, as well as a varying number of negative forms.

If the smears show only Gram-negative organisms, the material probably contains one or more of the following:

Gram-negative bacilli.	<ul style="list-style-type: none"> <li>B. coli group.</li> <li>B. typhosus group</li> <li>B. dysenteriae group.</li> <li>B. proteus.</li> <li>B. mucosus (capsulatus).</li> <li>B. pyocyaneus.</li> <li>B. influenza group.</li> <li>B. pertussis group.</li> <li>B. fusiformis.</li> </ul>	<ul style="list-style-type: none"> <li>Most frequently from intestinal tract.</li> <li>Most frequently from chest contents.</li> </ul>	Most frequently found, and some indication of their presence in history.
	<ul style="list-style-type: none"> <li>B. mallei.</li> <li>B. edematis (malignant edema).</li> <li>B. of symptomatic anthrax.</li> <li>B. pestis.</li> <li>B. of Morax-Axenfeld.</li> </ul>	Less frequently found, and generally a marked indication of their presence in history.	
Gram-negative cocci.	<ul style="list-style-type: none"> <li>Micrococcus intracellularis.</li> <li>Micrococcus catarrhalis.</li> <li>Micrococcus gonorrhoeae.</li> <li>Micrococcus melitensis.</li> </ul>	Generally marked indication of their presence in history.	
Gram-negative spirilla.	<ul style="list-style-type: none"> <li>S. cholerae and allied forms.</li> <li>Mouth spirals.</li> <li>Tr. pallidum.</li> <li>Sp. recurrentis.</li> </ul>	<ul style="list-style-type: none"> <li>Marked indication of presence of first form in history.</li> <li>Unimportant, unless indicated in history, when Tr. pallidum or Sp. recurrentis, respectively, should be looked for.</li> </ul>	

Old forms of any of the Gram-positive or Gram-amphophile organisms.

If only Gram-positive organisms are demonstrated, the material may contain one or more of the following:

Gram-positive bacilli.	<ul style="list-style-type: none"> <li>B. diphtheriae group.</li> <li>B. tetani (not often demonstrated in smears from lesion).</li> <li>B. tuberculosis group.</li> <li>B. leprae group.</li> <li>B. anthracis group.</li> <li>B. welchii and some other intestinal anaerobes.</li> </ul>	Generally marked indication of their presence in history.
Gram-positive cocci.	<ul style="list-style-type: none"> <li>Staphylococcus group.</li> <li>Streptococcus group (including pneumococcus and its variety, pneumococcus mucosus).</li> <li>Micrococcus tetragenus.</li> </ul>	Some indication of their presence in history.
Gram-positive spirilla.	None.	
Higher bacteria.	<ul style="list-style-type: none"> <li>Nocardia.</li> <li>Actinomyces.</li> </ul>	

Yeasts and molds, certain forms.

If organisms partially stained by Gram's method are demonstrated, the material may contain one or more of the following:

Amphophile.	Molds.
	Yeasts.
	Protozoa.
	Slightly known cocci and bacilli.

Certain slightly known streptococci and bacilli, older forms of any of the Gram-positive organisms.

**Regional Distribution of Microorganisms.**—From the different parts of the body the following more important organisms are found in the order of their probable frequency.

Fluids from serous membranes.	Meningeal (cerebrospinal).	Micrococcus intracellularis. Streptococcus (including pneumococcus) group. B. influenzae group.	Fluid generally cloudy with many leukocytes. Poliomyelitis fluid nearly clear, by transmitted light ground glass appearance.
	Pericardial and pleural.	B. tuberculosis group.	Fluid generally clear.
		Streptococcus (including pneumococcus) group.	Fluid may be cloudy.
		B. mucosus (capsulatus) group. B. influenzae group. B. tuberculosis group.	
Lungs.	Peritoneal.	B. coli group. Streptococcus group. B. tuberculosis group.	Fluid generally clear.
		Yeasts, molds, amebæ.	
		Streptococcus (including pneumococcus) group.	
		Micrococcus (including staphylococcus and diplococcus) group. B. mucosus (capsulatus). B. influenzae group. B. tuberculosis group. B. pertussis group. Nocardia. Yeasts, molds, amebæ.	
Nose and throat.		B. diphtheriae group. B. influenzae group. Streptococcus group. B. mucosus group. B. tuberculosis. Yeasts, molds, amebæ. Tr. pallidum.	
Sputum.		Same organisms as those found in the lungs, nose and throat.	
Eye.		Streptococcus (including pneumococcus) group. Group of hemoglobinophilic bacilli. Gonococcus group.	
		B. Diphtheriae group.	
		B. Morax-Axenfeld group.	
		B. tuberculosis group. Yeasts and molds.	
Feces.		B. coli (including B. fecalis alcaligenes and B. acidi lactici). B. typhosus group. B. dysenteriae group.	
		Gram-positive anaërobes. Yeasts, molds, amebæ.	
		B. tuberculosis.	
		Many forms whose importance has not been worked out.	
Urine.		B. coli group. Streptococcus (kidney). M. gonorrhæe.	
		B. typhosus.	
		B. tuberculosis.	
		Molds, flagellates.	

Pelvic organs.	{	M. gonorrhææ.
		Streptococcus.
	{	B. tuberculosis.
		Treponema pallidum.
	{	Certain anaërobæ.
		Many other forms probably much less important.
Blood.	{	Streptococcus (including pneumococcus) group.
		B. typhosus group.
	{	Trypanosome group.
		Group of malarial organisms.
	{	Sp. recurrentis.
Brain.	{	Meningococcus group.
		Strepto-pneumococcus group.
	{	Group of hemoglobinophilic bacilli.
		B. tuberculosis group.
	{	Tr. pallidum.
		Malarial organisms.
	{	Rabies organisms.
		Yeasts, molds, etc.

**Choice of Media.**—The following media should be used for the accompanying reasons:

Nutrient broth, for motility, morphology, and arrangement (chains, groups, etc.).

Potato for color and abundance of growth.

Peptone broth for indol.

Fermentation tube for anaërobæ, acidity and gas.

Nutrient agar and gelatin.	{	(a) Poured plates for isolated colonies (dilutions according to the number of organisms seen in smears). (Blood-agar if pneumococcus or streptococcus indicated.)
		(b) Streaked plates for surface colonies. (Blood-agar if influenza bacilli are indicated.)

**Sputum Washing.**—Some of the associated bacteria found in the expectoration come from the diseased areas of the lungs, while others are merely added to the sputa as it passes through the mouth or are developed after gathering. To endeavor to separate the one from the other we wash the sputa. The first essential is that the material is to be washed within a few minutes, and certainly within an hour after being expectorated. If a longer time is allowed to intervene, the bacteria from the mouth will penetrate into the interior of the mucus, and thus appear as if they came from the lungs. Sputum treated twenty-four hours after its expectoration is useless for examining for anything except the tubercle bacillus. A rough method is to pour some of the specimen of sputum to be examined into a convenient receptacle containing sterile water, and withdraw, by means of a sterilized platinum wire, one of the cheesy masses or thick "balls" of mucus. Pass this loop five times through sterile water in a dish; repeat the operation in fresh water in a second and third dish. Spread what remains of the mass on cover-glasses and make smear preparation; stain and examine. With another mass inoculate aseptic bouillon in tubes and agar in plates.

When we wish thoroughly to exclude mouth bacteria, a lump of the sputum raised by a natural cough is seized by the forceps and transferred to a bottle of sterile water and thoroughly shaken; it is then removed to a second bottle of bouillon and again thoroughly shaken. From this it is passed in the same way through four other bottles of bouillon. A portion of the mass is now smeared over cover-glasses, and the rest inoculated in suitable media, such as agar in Petri dishes, and aseptic fluid bouillon in tubes. If desired, the bacteria washed off in the different washings are allowed to develop.

## CHAPTER VII.

### THE RELATION OF MICROÖRGANISMS TO DISEASE.

IN preceding chapters we have considered the growth of microorganisms for the most part in dead organic substances. Now we have to consider their growth and the introduction of the foreign protein of their protoplasm and the poisons produced by them in the living host and the resulting reactions. While it is true that there is a great difference between living and dead matter, and that, therefore, the living animal cannot be looked upon as merely a quantity of special material to be used for food for growth, still, in a very real sense, we are warranted in considering the infected living body as a food mass more or less favorable for the growth of microorganisms. The difference is that besides the active ferments, chemical substances, temperature, and conditions inherent in the fluids and cells of the living body, microorganisms have also to reckon with the constant production of new substances by the living cells of the invaded organism, which may be antagonistic to them as well as by the presence naturally of similar substances. In the production of disease, therefore, four main factors are involved, viz., on the part of microorganisms, the power to elaborate poison or proteins that may be split by ferments to yield poisons, and the ability to multiply; on the part of the body, its initial condition depending on the nature of its fluids and the degree of sensitiveness of its cells to the poisons of the microbe, and its reaction to attack, as shown in the response of the cells which absorbed the poisons or proteins of the invading germs to develop substances which neutralize the poisons or attack the invading bacteria or protozoa. No known variety of microorganisms has, in very small numbers, the ability to produce enough poison to do appreciable injury to the body, nor is it probable that there is any variety which, if it multiplied in the body to the extent that some pathogenic bacteria are capable of, would not produce appreciable disease. The division of parasitic microorganisms, therefore, into pathogenic and non-pathogenic forms is a relative one, just as is the broader division into saprophytic and parasitic forms (see p. 51).

That different strains of the same species of microorganisms have different toxin-producing powers under what appear to be practically the same conditions is illustrated by the following fact: If we take specimens of diphtheria bacilli from three different cases of diphtheria, we sometimes find that on growing them for several days in suitable bouillon one culture will have produced poison in the fluid to such a degree that a drop suffices to kill a large guinea-pig; the second, grown in a similar manner, will kill another animal of the same size with half a drop; while the third will kill with one-tenth of a drop.

The cultivation of the tetanus bacillus furnishes some interesting facts



which illustrate the complicated ways in which the growth of varieties of bacteria are hindered or assisted. The tetanus bacillus, when placed in suitable media, will not grow except in the absence of oxygen; but place it under the same conditions, together with a microorganism which actively assimilates oxygen, and the two in association will grow in the presence of air. As a rule, when tetanus bacilli are driven into the flesh by a dirty nail or blank cartridge plug, aerobic bacteria are driven in also and so help to further infection by using up the free oxygen, thus introducing an anaerobic environment.

The influenza bacillus is another example of the special requirements of certain bacteria. On media it will thrive in pure culture only in the presence of hemoglobin, while with certain other bacteria it will grow luxuriantly without hemoglobin.

It is evident from these and other facts that for each variety of organism there are special conditions requisite for growth, and that a temperature, degree of acidity, kind of food, supply of oxygen, etc., suitable for one may be utterly unsuitable for another; that, still further, when two organisms grow together one may so alter some of these conditions as to render an unsuitable one suitable, and *vice versa*. We can understand in a measure from the above incomplete statement how the different types of microbes do not grow equally well in every variety of host, or even in the different tissues of the same host; and we begin to understand the variations in intensity of disease. When we learn further of the specific and varying degrees of reaction on the part of the host to the invading microorganisms we begin to realize that the result may be either the death of the invader or of the invaded, or the ability of one to become accustomed to the other and so to live together. In this outcome the invader either loses the ability to produce disease in any host, thus becoming a harmless parasite (commensal), or retains the same pathogenic properties. In the latter case the primary host is termed "a germ carrier."

**Manner in which Microorganisms Produce Injury.**—The pathogenic power of microbes may be due to one or more of three factors: mechanical injury, the use of tissue as food, and the irritation and destruction of tissue by poisons. Serious mechanical injury is produced only when germs exist in such enormous numbers or are bunched so as to interfere mechanically with the circulation or, together with fibrin, to cause minute thrombi, and later emboli which finally produce infarction and abscesses in different parts of the body. Such mechanical injury is different from that produced by non-poisonous substances, however, since most microbes contain or excrete chemical substances which are directly poisonous or become so after being split by ferments. Some portion of the protoplasm of almost every variety of bacteria acts as an irritant to tissues and combines with some of the substance of some of the body cells, and the protoplasm of most exerts a positive chemotaxis.

The poisonous products can often be separated from the fluid in which the organisms have grown, or they can be extracted from the microbes. Injected into animals these products cause essentially the same cellular lesions as are produced by the organisms when they develop in the animal body.

**Limitations of Pathogenic Action.**—As we have intimated the power of the microbe to produce disease is limited by the numbers introduced, by the ability to grow, by the ability to produce poisons both specific (toxins) and non-specific, and by the general and specific protective powers of the host.

**Influence of Quantity in Infection.**—With pathogenic microbes the number introduced has an immense influence upon the probability of infection taking place.

If we introduce a few bacteria into a culture medium containing some fresh human blood or serum it is probable that they will all die because of the presence of sufficient bactericidal substance in the blood to destroy them; whereas if a greater number is introduced there will be at first a great diminution of these, those that die having combined with the bactericidal substances in the serum which neutralizes them; then those bacteria which survive begin to increase, and soon they multiply enormously. The same is true for parasitic microbes in the body. A few only gaining entrance, all may die; a larger number being introduced, some may or may not survive; but if a still greater quantity is injected it is almost certain unless the animal is immune that there will be some surviving members, which will begin to multiply and excite disease. The ability of the microbe to grow at the expense of a host is called its virulence, while its ability to produce specific toxins is called its toxicity.

**Adaptation of Microbes to the Soil upon which They are Grown.**—Those organisms which grow both in living and dead substances vary from time to time as to their readiness to develop in either the one or the other. As a general rule, bacteria grown in any one medium become more and more accustomed to that and other media more or less analogous to it, while, on the other hand, they are less easily cultivated on media widely different from that in which they have developed. Thus we had a culture of tubercle bacilli which, after having grown for three years in the bodies of guinea-pigs, would grow only with great difficulty on dead organic matter, while a duplicate culture grown for the same time on bouillon, is scarcely able to infect a guinea-pig. From the same stock, therefore, two varieties have developed, the one having lost and the other gained in ability to develop as a parasite.

Parasitic microorganisms not only gradually adapt themselves to certain species of animals, but to certain circumscribed areas of the body. Thus the diphtheria bacilli grow chiefly upon the mucous membranes of the respiratory tract, but do not develop in the blood or in the subcutaneous tissues. The cholera spirilla develop in the inflamed intestinal mucous membrane, but do not grow in the respiratory tract, blood, or tissues. The malarial parasites develop in the human body chiefly in or on the red blood cells.

Other microbes find, indeed, certain regions especially suitable for their growth, but under conditions favorable for them are capable of developing in different locations. Thus, the typhoid bacillus grows

most luxuriantly in the Peyer patches and mesenteric glands, but also invades the blood, spleen, and other regions. The tubercle bacillus often remains localized in the apex of a lung or a gland for years, but may at any time invade many tissues of the body. The gonococcus finds the mucous membrane or the genito-urinary tract most suitable for its development, but also frequently is capable of growth in the eye and peritoneum and sometimes in the general circulation. The pneumococcus develops most readily in the lungs, but also invades the connective tissues, serous membranes, and the blood. The malarial protozoa grow not only in human red cells, but also in the cells of the stomach and the salivary glands of the mosquito.

Many microorganisms of no apparent virulence never invade the body but grow more or less as parasites on the surface of skin and mucous membranes; while some varieties may be carried passively by the blood or lymph to various organs of the body, notably the spleen and the lymph nodes and there lodge for a variable time without doing demonstrable harm. Whether any of these by slight accumulative action throughout a lifetime help to produce certain chronic conditions is not known.

**Variation in Degree of Virulence and Toxicity Possessed by Microbes.**

—Microbes differ, as has already been stated, as to the ease and rapidity with which they grow in any living nutritive substance—that is, as to their virulence—and as to the amount of poison they produce. Both of these properties not only vary greatly in different members of the same species, but each variety of organism may, to a certain extent, be increased or diminished in virulence and toxin production. The septicemic class of bacteria when grown in the body fluids seem gradually to develop protective substances and to cease to produce as much susceptible material in their own bodies. Or they may develop a protecting capsule so as to present less substance having affinity for the bactericidal bodies of the blood, and thus to become less vulnerable.

With those bacteria whose virulence is great, it requires only very few organisms to invade the host and these produce disease almost as quickly as a million, allowance being made for the short time required for the few to become equal in number to the million. At the other extreme of virulence, however, many millions may have to be introduced in order to overcome the body resistance and to permit the development of any new organisms in the body. With these bacteria, therefore, by regulating the number of organisms introduced, we may produce no effect whatever, or a local effect, or a general septicemia.

Somewhat distinct, again, from that class of microorganisms which multiply rapidly are those like the tubercle and leprosy bacilli, which, while surely developing infection, increase more slowly. Here increase of virulence is shown, as before, by the production of disease through the introduction of very small numbers into the body, but increase in rapidity of development cannot progress except to within certain limits. A single streptococcus may, through its rapid multiplication, produce death in eighteen hours; a single tubercle bacillus,

on the other hand, cannot produce sufficient numbers in less than two weeks. The virulence of the septicemic class of bacteria is not at all the same when measured in different animals, and it is largely for this reason that the virulence in test animals does not usually correspond with the severity of the case from which the organism was derived.

**Experimental Increase and Decrease in Toxicity and Virulence.**—

The power to produce toxin can be taken from microbes by growing them under adverse circumstances, such as cultivation at the maximum temperature at which they are capable of development. Some microbes are easily attenuated; others are robbed of their virulence only with great difficulty. Increase of toxin production is more difficult, and it is only possible to obtain it to a certain extent. The usual method employed is the frequent replanting of cultures. But with all our efforts we are usually only able to restore approximately the degree of toxin formation which the cultures originally possessed. On the other hand, the attempt to make microbes grow on any nutritive substance, living or dead, is more successful. The streptococcus from erysipelas and the pneumococcus from pneumonia are typical of this class of organisms. Inoculate a rabbit with a few streptococci obtained from a case of human sepsis, and, as a rule, no result follows; inject a few million, and usually a local induration or abscess appears; but if 100,000,000 are administered, septicemia develops. From this rabbit now inoculate another, and we find that a dose slightly smaller suffices to produce the same effect; in the next animal inoculated from this, still less is required, and so on, until in time, with some cultures, a very minute number will surely develop and produce death. With other cultures increase in virulence does not take place. The same increase in virulence can be noted when septic infection is carried in surgery or obstetrics from one human case to another.

The nature of some of the more important microbial poisons will be discussed in the next chapter.

**Mixed Infection.**—The combined effects upon the tissues of the products of two or more varieties of pathogenic microbes, and also of the influence of these different forms on each other, are of great importance in the production of disease. The infection from several different organisms may occur at the same time, or one may follow the other or others—so-called secondary infection. Thus, an abscess is often due to several forms of pyogenic cocci. If a fresh wound is infected from such a source, the inflammation produced will probably be caused by all the varieties present in the original infection. Peritonitis following intestinal injuries must necessarily be due to more than one variety of organism. Thus, whenever two or more varieties of bacteria are transferred to a new soil, mixed infection takes place if more than one is capable of developing in that locality.

Forms of infection which are allied to both mixed and secondary infection are those occurring in the mucous membranes of the respiratory and digestive tract. In these situations pathogenic microbes of slight virulence are always present even in health. Thus, in the

upper air passages there are usually found streptococci, staphylococci, and pneumococci. When through the invasion of one or several infective agents, as the diphtheria bacillus or the virus of smallpox or scarlet fever, the epithelium of the mucous membrane of the throat is injured or destroyed, the pyogenic cocci already present are now enabled to grow in this diseased membrane, to produce their poison, and even to invade deeper tissues. The intestinal mucous membrane is invaded in a similar way by the colon bacilli and other organisms after injury by the typhoid or dysentery bacilli or cholera spirilla. Generally speaking, all inflammations of the mucous membranes and skin contain some of the elements of mixed infection. Blood infection, on the other hand, is usually due to one form of microbe, as even when several varieties are introduced, only one, as a rule, is capable of development. The same is true to a somewhat less extent of inflammation of the connective tissue. The additional poison given off by the associated microbes aid infection by the primary invaders by causing a lowering of the vital resistance of the body. In some cases the secondary infection is a greater danger than the primary one, as pneumococcic bronchopneumonia in laryngeal diphtheria or streptococcic septicemia in scarlet fever and smallpox.

Microörganisms are also at times directly influenced by the products of associated organisms. These may affect them injuriously, as for example, the pyogenic cocci in anthrax; or they may be necessary to their development, as in the case of anaërobic bacteria. Not infrequently the tetanus bacilli or spores would not be able to develop in wounds were it not for the presence of aërobic bacteria introduced with them. This is shown outside the body, where tetanus bacilli will not grow in the presence of oxygen unless aërobic bacteria are associated with them. Again, it is found that the association of one variety with another may increase its virulence. Streptococci are stated to increase the virulence of diphtheria bacilli, but here it is probably the loss of resistance of the tissues because of the streptococcic poison. On the other hand, the absorption of the products of certain bacteria immunizes the body against the invasion of other bacteria, as shown by Pasteur that attenuated chicken-cholera cultures produced slight immunity against anthrax. In intestinal putrefaction harmless varieties of bacteria may be made to crowd out dangerous ones.

**Protective Powers of Host.**—Living beings are protected naturally against invasion from germs by three general defences: (1) by mechanical means, such as tough envelopes unsuited for growth or penetration; (2) by chemical substances which either kill the organisms or render harmless their pathogenic products; and (3) by the taking up of the organism by certain host cells (phagocytosis).

**Importance of Location of Point of Entry of Organisms.**—Most microbes cause infection only when they gain access to special tissues and must therefore enter through certain portals. This fact is of immense importance in the transmission or prevention of disease. Thus, for example, let us rub very virulent streptococci, typhoid bacilli,

or diphtheria bacilli into an abrasion on the hand. The typhoid bacillus produces no lesion, the diphtheria bacillus but a very minute infected area, but the streptococcus may give rise to a severe cellulitis or fatal septicemia. Now place the same bacteria on an abrasion in the throat. The typhoid bacillus is again harmless; the diphtheria bacillus produces inflammation, a pseudomembrane, and toxemia, and the streptococcus causes an exudate, an abscess, or a septicemia. Finally, introduce the same bacteria into the intestines, and now it is the typhoid bacillus which produces its characteristic lesions, while the streptococcus and diphtheria bacillus are usually innocuous.

If we tried in this way all the parasitic organisms we would find that certain varieties are capable of developing, and thereby exciting disease only on the mucous membrane of the throat, others of the intestine, others of the urethra; some develop only in the connective tissues or in the blood; while others, again, under favorable conditions, seem able to grow in or upon most regions of the body.

**Tissue Characteristics Influencing the Entrance and Growth of Microbes.**—**The Skin.**—The skin is a poor soil for bacteria and is a great protection against the penetration of microorganisms. When they do penetrate, it is through the diseased glands, or more often through some unobserved wound. The bacterial toxins are, when at all, absorbed to a slight extent through the skin.

There is an apparent exception to the above statements in the fact that the pyogenic staphylococci and sometimes the streptococci exist upon the skin or in it between its superficial horny cells, some exceptional circumstances, such as wounds, or burns, being required to allow the organisms to penetrate deeper. The cutaneous sweat glands, and the hair follicles with their appended sebaceous glands, may allow entrance of infection, as various incidents may lead to the introduction and retention of virulent microorganisms. When this occurs the retained products may lead to necrosis of the epithelium and thus allow the bacteria to penetrate to the deeper tissues. The secretion of the sebaceous glands appears to be little, if at all, bactericidal, but the perspiration, on account of its acidity, is slightly so. A species of protozoa (*Leishmania*) is found frequently in certain skin lesions in the tropics.

**Subcutaneous Connective Tissues.**—Many microbes cannot develop in the connective tissues and others produce a milder infection there than elsewhere. Others develop readily. The tissue fluids have microbicidal properties similar to but less in amount than the blood.

**Blood and Lymph.**—Both of these fluids have normally certain protective powers, some of which vary with age and other conditions. They will be considered minutely farther on.

**The Serous Membranes.**—The endothelial cells lining these membranes are able to a certain extent to act as phagocytes for some bacteria. But certain organisms of a low grade of virulence, *e. g.*, some streptococci that are taken up in the joint linings, may grow and produce

chronic inflammations, and others of greater virulence may excite acute infection.

**The Mucous Membranes.**—The moist condition of the surface of the membranes and the frequent contact with irritating substances render them liable to microbial infection. Organisms, such as the pneumococci and streptococci, reproducing themselves here become somewhat attenuated. The mucous membranes are protected by the cleansing produced by the flow and slight germicidal action of their secretion. In infancy the membranes are readily infected by gonococci and later by pneumococci, by influenza bacilli and by others. The mucous membranes of the nasal cavity are somewhat cleansed by the nasal secretion. The deeper portions of the nasal cavity are usually the seat of streptococci and other bacteria, while the extreme anterior portion contains saprophytic bacteria from the air. The mouth in a person in health is cleansed by the feebly bactericidal saliva. When the teeth are decayed many varieties of microbes abound. Many of these are difficult to cultivate. The bacteria, such as the diphtheria bacilli, streptococci, etc., invade the mucous membrane of the tongue or mouth comparatively seldom.

The tonsils with their crypts are usually the seat of the pyogenic cocci and are readily infected by the diphtheria bacilli and others. Whether the absolutely intact epithelium allows the passage of these bacteria is disputed, but the probability is that it does. With slight pathological lesions usually present it undoubtedly does.

**Muscles.**—The muscle fibers themselves are not often primarily attacked by microbes. The germs pass them and grow in the blood and lymph vessels and in the connective tissue, and the changes in the muscle fibers are chiefly degenerative and secondary. Some animal parasites, however, select the muscle fibers as a favorite site for growth, and here they may be encysted by a wall of formative connective-tissue cells.

**The Lungs.**—Most inhaled organisms which pass the larynx are caught in the bronchi. Many of these are gradually removed by the ciliated epithelium. Both the alveolar epithelial cells and the leukocytes which enter the air sacs and bronchioles have been shown to take up bacteria. The normal lung is therefore rapidly freed of saprophytic and many parasitic bacteria. When subjected to deleterious influences, such as exposure to cold, the lung tissues seem to lose their protective defences and become subject to infection.

**The Stomach.**—The pure gastric juice, through the hydrochloric acid it contains, is able to kill most non-spore-bearing organisms in a short time, but because of neutralization through food, or because the microbes are protected in the food, many of them pass into the intestines. Tubercle, typhoid, colon, and dysentery bacilli, when fed by the mouth with food, readily pass beyond the stomach. Certain acidophilic bacteria, as well as yeasts and torulæ, seem to grow in the gastric secretion; these are largely non-pathogenic. Perforation of the stomach is usually followed by peritonitis, because of the irritant

effect of the gastric juice and the bacteria which were temporarily present in the stomach. The gastric juice alters tetanus and diphtheria toxins. The toxicity of some poisons, such as occur in decayed meat, are not destroyed. The stomach is exceptionally free from bacterial inflammations.

**Intestines.**—The bile is feebly germicidal for some organisms, but, on the whole, the intestinal secretions have little or no germicidal power. The number of microbes increases steadily from the duodenum to the head of the colon, and diminishes slightly from the upper to the lower end of the colon. The pancreatic juice destroys many of the toxic microbial products. The presence of the bacilli of the colon group, of streptococci, etc., does not often lead to any inflammatory condition in the normal intestines of healthy persons. In children suffering from the prostrating effects of heat they are apt to excite inflammatory changes. Even pathogenic bacteria, such as the typhoid, dysentery, and tubercle bacilli, may pass through the whole length of the healthy intestines without inciting inflammations. Slight lesions aid the passage of bacteria to the deeper structures. Tubercle bacilli and other pathogenic bacteria may pass through the intestinal wall to the lymph and cause distant infection without leaving any trace of their passage. Non-pathogenic protozoa are frequently found in the intestines, and, in tropical countries, pathogenic forms are also frequent.

**Bones.**—Bones are infected through the circulation, except in case of exposure from a direct injury. Hence the periosteum and the bone marrow are first and chiefly affected. The bony structure may secondarily become necrotic and absorbed. An infection once established in bone tissues is difficult to check. In this way pyorrhea and abscesses form about the teeth, due to several different organisms, and necrosis of bones occur in various parts of the body due to tubercle bacilli, syphilitic infection, staphylococci, actinomycosis, or, less frequently, certain other organisms.

**Specific Antibody Protection.**—Naturally most human beings as they grow to maturity have suffered from infectious diseases or have been carriers of pathogenic bacteria in the mucus of the respiratory organs and the intestines and some of these have been absorbed. They have then developed substances in their tissues that have a specific neutralizing action on many pathogenic bacteria. The nature of these substances and the way in which they may be acquired artificially will be taken up later.

**Pathological Effects Produced.**—According to the type of attacking microbe we may have either microbial or toxic effects which may be either local or general or both. When a microbe invades and goes throughout the body it is spoken of as an invasive microbe and the condition produced is spoken of as microbemia, septicemia (bacteremia in the case of bacterial invasion). If the microbes grow in disseminated foci throughout the body the condition is called pyemia. When the germs grow only at the point of entry and the toxin is absorbed and



does the injury, they are spoken of as toxic organisms, and the condition produced is called toxemia.

**Local Effects Produced.**—The local effects of the microbial poisons and proteins upon the cells give rise to the various kinds of inflammation, such as serous, fibrinous, purulent, croupous, hemorrhagic, necrotic, gangrenous, and, finally, proliferative. Such organisms incite specific forms of inflammation along with those common to many organisms; others produce, so far as we can detect, no peculiar form of lesions.

**General Symptoms Produced.**—Fever is produced, under favorable conditions, by all microbial proteins. A requisite is that sufficient proteins be absorbed; on the other hand, they must not be absorbed with such rapidity as to overwhelm the infected host, for a moderate dose may raise the temperature, while a very large dose lowers it, as occurs sometimes when a very large surface, such as the peritoneum, is suddenly involved. As Vaughan and others have shown, these foreign proteins have to be acted upon by the body ferments before they exert their poisonous effect.

With few exceptions the microbial proteins produce an increase in the number of leukocytes and a lessening in the amount of hemoglobin in the blood. In uncomplicated infection with typhoid bacilli there is a leukopenia. The polynuclear leukocytes are usually increased in varying proportions in bacterial infections. The red blood cells are directly injured by a number of bacterial substances. The deleterious effects on the nutrition are partly due to the direct effect of the poison and partly to the diseased conditions of the organs of the body, such as the spleen, kidney, and liver. Degeneration of the nerve cells is frequently noticed after infectious diseases; especially is this true of diphtheria. Several bacterial poisons have been found to produce convulsions; the best example of this is the tetanus toxin. In protozoan infections the mononuclear leukocytes and the eosinophiles are chiefly increased.

Vaughan has described the effects produced when a fatal dose of a living virulent organism is injected into the peritoneal cavity of a guinea-pig as follows: For a period of time which usually varies from eight to twelve hours the animal remains apparently normal. Its temperature may fluctuate slightly, but not beyond the normal limits. This is the period of incubation and varies within certain time limits, but within these it is fairly constant. During this time the bacteria are multiplying enormously in the animal body. They are converting animal proteins into bacterial proteins. This is largely a synthetic or constructive process. The soluble proteins of blood and lymph are built into the cellular proteins of the bacteria. There is no liberation of the protein poison and consequently no disturbance in the well-being of the host. During the period of incubation of an infectious disease the invading organism supplies the ferment, the soluble proteins of the animal body constitute the substrate, the process is constructive, simple proteins are built into more complex ones, no protein poison is

liberated, and no recognizable symptoms mark the progress of the infection. Still, in the development of the phenomena of infection, the period of incubation is critical, and the rate at which the infecting virus multiplies during this time is an important factor in determining the final outcome. The more virulent the virus, the more rapidly does it multiply and this means a larger amount of animal protein converted into bacterial protein.

Somewhat abruptly there is a change in the behavior of our inoculated guinea-pig. The hairs behind the ears begin to stand out and soon the entire coat becomes rough. It no longer eats, but retires to one corner of the cage and seems to be in distress. Slight pressure over the abdomen elicits evidence of pain and the temperature begins to fall and continues to do so until death. In the case of recovery, a rise of temperature is the first evidence of improvement.

This somewhat abrupt change in the condition of the animal marks the end of the period of incubation and the beginning of the active disease. The animal cells have become sensitized and are now pouring out a specific ferment which digests the bacterial proteins. In the active stage of the disease the animal cells supply the ferment, the bacterial proteins constitute the substrate, complex cellular proteins are split into simpler bodies, the process is analytic and destructive, the protein poison is liberated, the symptoms of disease develop, and life is placed in jeopardy.

**The Elimination of Microbes and Their Products from the Tissues.**—Those microbes that survive the protective defenses of the host enumerated above, after having gained access to the blood and other tissues may be more or less quickly conveyed to the body surfaces by several channels, or they may remain located at various points along the channels of elimination, such as the gall-bladder, from which they may be gradually discharged to the surfaces of the host and thence to the outside world. Such collections of germs, though temporarily at least non-pathogenic for the host, or carrier (see below), may be quite pathogenic for other individuals.

**Channels of Elimination.**—Comparatively few microbes pass from the blood through the mucous membranes, either with or without the aid of "wandering cells." Large quantities of poisonous products of the germs on the contrary may be eliminated in this way, especially through the kidneys by the urine. Whether bacteria pass from the blood by the sweat is a mooted point. The skin is always the seat of the staphylococcus and frequently of other bacteria, so that it is difficult to determine in any given case the origin of the bacteria found in the sweat. The passage of bacteria through the breast is important, from the fact that milk is so largely used as food. Observers have reported the finding of tubercle bacilli in cows' milk when the gland itself was intact and the animal tuberculous. The fact that tubercle bacilli swallowed with the sputum are passed alive in the feces explains the frequent occurrence of bacilli in the milk of cows without udder tuberculosis because of the contamination of the milk with manure. They are undoubtedly present,

however, in the milk of some animals in which tuberculous disease of the gland could not be demonstrated. In these cases lymph glands adjacent to the udder are usually infected. The finding of streptococci and staphylococci is due probably in the majority of cases to the infections taking place as the milk is voided, for the epithelium at the outlet of the lacteal ducts is always infected with staphylococci, and frequently with streptococci, which have often been received from the mouth of the sucking infant. The cow's udder may be infected from the hands of the milker. Epidemics of septic sore throat have been traced to such cause.

**The Dissemination of Disease.**—The spread of infection is influenced by: 1. The number and species of animals subject to infection.

Many human infectious diseases do not occur in animals, and many animal infections are not found in man. Thus, so far as we know, gonorrhea, syphilis, measles, smallpox, typhoid fever, etc., do not occur in animals under ordinary conditions; while tuberculosis, anthrax, glanders hydrophobia, and some other diseases are common to both man and animals.

2. The quantity of the infectious material and the manner in which it is thrown off from the body.

In diphtheria, typhoid fever, cholera, pulmonary tuberculosis, septic endometritis, influenza, and gonorrhea, enormous numbers of infectious bacteria are cast off through the discharges from the mouth, intestines, and genito-urinary secretions, causing great danger of infection. On the other hand, in tuberculous peritonitis, streptococcic meningitis and endocarditis, gonorrheal rheumatism, and the like there is little or no danger of infecting others, as few or no bacteria are cast off.

3. The resistance of the infectious organisms to the deleterious effects of drying, light, heat, etc.

In this case the presence or absence of spores is of the greatest importance. The spore-bearing bacilli such as tetanus and anthrax, being able to withstand destruction for a long time, retain their power of producing infection for months or even years after elimination from the body. The bacteria which form no spores show great variation in their resistance to outside influences. Some of these, such as the influenza bacilli and the gonococci, the virus of syphilis and hydrophobia, are extremely sensitive; the pneumococci, cholera spirilla, glanders bacilli, etc., are a little harder, then follow the diphtheria bacilli, and, after them the typhoid and tubercle bacilli and the staphylococci. Yeasts, molds, and protozoa, produce resisting spores, which, however, are not as highly resistant as most bacterial spores.

4. The ability or the lack of ability to grow outside of the infected tissues.

Such bacteria as the pneumococcus, tubercle, influenza, diphtheria, glanders, and leprosy bacilli do not develop, as far as we know, outside of the body under ordinary conditions. Under exceptional circumstances, as in milk, some may develop. Others, again, such as the

streptococcus and staphylococcus, typhoid and anthrax bacillus, the cholera spirillum, and some anaërobes, may develop under peculiar conditions existing in water or soil.

While for the pathogenic bacteria, as a rule, the saprophytes met with in the soil and water are antagonistic, yet in some cases—and especially is this true of the anaërobic bacteria—they are helpful.

5. **Germ Carriers.**—In human carriers microörganisms develop in or upon some portion of the skin or mucous membrane, either after or before disease, and without causing infection. As complete a knowledge as possible of this commensal development in man of pathogenic microbes is necessary if we are to combat the spread of infection. In the superficial layers of the epithelium and on the surface of the skin we find the different pyogenic cocci, which are capable of infecting a wounded or injured part or causing inflammation in the glands. Acne, the pustules in smallpox, the pus on a burned surface, boils, etc., all are aided by these pyogenic cocci. In surgical cases the skin has to be as thoroughly disinfected as possible to prevent the formation of stitch-hole abscesses and wound suppuration.

In the secretion of the mucous membrane covering the pharynx and nasopharynx there is always an abundance of microbes. In throats examined in New York City, streptococci, staphylococci, and pneumococci are found in almost every instance, and even in the country they are often present. In the anterior nares there are fewer parasitic bacteria than in the posterior portions. Many other varieties of bacteria, such as the meningococci and the influenza bacilli, are probably often present in small numbers. In those constantly in contact with cases of diphtheria, and in those convalescent from diphtheria, virulent diphtheria bacilli are frequently found in the throat.

After convalescence from typhoid fever, from 1 to 3 per cent. remain bacillus carriers for months or years. The bacilli continue to develop in the bile passages and are passed with the feces.

Certain pathogenic protozoa may be carried for some time in the intestines of man.

**Lower Animals.**—The lower animals, as a rule, do not retain in their bodies bacteria pathogenic for human beings, but as direct carriers of bacterial infection they are important factors. Flies and other insects may convey organisms which are simply attached to their feet or other surfaces of their bodies (accidental carriers). Biting insects especially, such as fleas, ticks, bed-bugs, lice, flies, and mosquitoes, are of special danger in certain protozoan infections, since these insects act as intermediate hosts in these cases. (See Protozoan Diseases.)

**Microbal Auto-infection.**—When the intestinal canal is injured, or its action hindered by strangulation, etc., *B. coli* or other bacteria ordinarily harmless may pass through the injured walls and cause peritonitis or general infection. Under certain conditions, as during the debility due to hot weather, the bacteria in the intestines cause, through their products, irritation, and in children even serious intestinal inflammation. Long after an acute gonorrhea has passed gonococci may

remain in sufficient numbers to cause a new inflammation or produce infection in others. A cystitis may run on chronically for years, and then suddenly become acute or spread infection to the kidneys. A persistent gonorrheal vaginal infection may lead to a gonorrheal endometritis, or peritonitis or salpingitis, under suitable conditions. The staphylococci in the skin and the colon bacilli and pyogenic cocci in the fecal discharges may also be carried into the bladder and uterus and produce septic infection. Persons carrying diphtheria bacilli in their throats or typhoid bacilli in their gall-bladder may, under predisposing conditions, develop diphtheria or typhoid fever. Blind abscesses at the roots of the teeth or elsewhere may be the cause of a slow dissemination of pyogenic organisms to the joints, the heart and other parts of the body. Evidence has been accumulating during the past few years through the work of Billings, Rosenow and others of the important part played by these *focal infections* in chronic diseases.

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## CHAPTER VIII.

### THE NATURE OF THE SUBSTANCES CAUSING DISEASE: MICROBAL POISONS.

MICROÖRGANISMS, like cells of the higher plants and animals, are composed of chemical substances of many types. So far as is known, only those of a protein character are concerned in the production of disease. The proteins of each species are in part at least specific for that species and when such a foreign protein is introduced into the tissue of an animal without previous digestion by the intestinal juices more or less disturbance may be produced. This disturbance may be due partly, according to one hypothesis, to the splitting of these proteins by ferments in the host fluids and cells and, partly, according to another hypothesis, to the deranging of the balance between the ferments and anti-ferments of the blood.

Besides containing protein having the general characteristics of a foreign protein, some of the bacteria (*e. g.*, *B. cholerae*, *B. dysenteriae*) contain proteins which are directly toxic, somewhat similar, for instance, to certain poisons in plants such as the castor oil bean which contains proteins, which have the characteristics of general protein and, in addition, contains ricin which is a peculiar irritant poison. All of these intracellular proteins are by some grouped as endotoxins and this without discrimination as to whether the protein is distinctly toxic or simply has the general qualities of a foreign protein.

A few microörganisms (*e. g.*, *B. diphtheriae*, *B. tetani*) also secrete specific poisons which are soluble and can be compared with the poison given off by the cells of the salivary glands of the rattlesnake. These extracellular poisons are called exotoxins, or soluble toxins.

A further and very important difference between these two types of poisons is that when members of the first type, the intracellular poisons, come in contact with the cells of the body, substances (antibodies) are produced which unite with these intracellular poisons in certain complex ways (discussed below) without directly neutralizing their deleterious effects; while poisons of the second type, the extracellular poisons, stimulate the cells brought in contact with them to produce directly neutralizing substances which unite with the extracellular poisons in definite proportions and render them harmless.

It will be valuable to consider some of the characteristics of these two types of microbial proteins in more detail and also some theories of their activity in the body of an infected person.

**Vaughan's Explanation of the Action of Intracellular Poisons.** (Endotoxin.) —“(a) The infective bacteria, taking the colon, typhoid, tubercle, and the pneumococcus as types, contain an intracellular poison. This is not a toxin;

it is not specific; it produces no antibody when injected into animals. (b) These bacteria elaborate no soluble toxin or poison. In old cultures there may be a trace of poison, but this results from the autolysis of the cells and is not a cellular secretion. (c) This poison is a group in the protein molecule, and can be obtained in soluble form only after cleavage of the cellular proteins which may be accomplished by superheated steam, dilute acids, or alkalis. (d) It exists in all true proteins, in pathogenic and non-pathogenic bacteria, and in vegetable and animal proteins. (e) It may result from the cleavage action of proteolytic ferments. (f) In most vegetable and animal proteins the poisonous group is neutralized by combination with non-poisonous groups; consequently such proteins have no poisonous action until they undergo molecular disruption. (g) This primary group is poisonous because of the avidity with which it combines with secondary groups in the proteins of the animal body. (h) The specificity of proteins lies in their secondary, non-poisonous groups. It is in these that one protein chemically and biologically differs from another. (i) Biological relationship among proteins is determined by the chemical structure of their molecules. There are as many kinds of proteins as there are kinds of cells. (j) The symptoms of the infections differ chiefly on account of the organ or tissue in which the virus accumulates and where it is split up and its poison liberated. (k) The ferment which causes the cleavage of the bacterial proteins in the different infectious diseases is specific."

**Theories as to the Nature of Extracellular Toxins and of Their Union with Antitoxins.**—From a large number of carefully conducted experiments with the toxin and antitoxin of diphtheria, Ehrlich has formulated a theory. This theory has undergone several modifications since it was first proposed, and it is difficult to give an exact statement of its present status. Generally speaking, however, in condensed form its essential points are as follows:

Toxins and antitoxins neutralize one another after the manner of chemical reagents. The chief reasons for this belief lie in the observed facts: (a) that neutralization takes place more rapidly in concentrated than in dilute solutions, and (b) that warmth hastens and cold retards neutralization. From these observations Ehrlich concludes that toxins and antitoxins act as chemical reagents do in the formation of double salts. A molecule of the poison requires an exact and constant quantity of the antitoxin in order to produce a neutral or harmless substance. This implies that a specific atomic group in the toxin molecule combines with a certain atomic group in the antitoxin molecule.

The toxins, however, are not simple bodies, but easily split into other substances which differ from one another in the avidity with which they combine with antitoxin. These derivatives Ehrlich calls prototoxins, deuterotoxins, and tritotoxins.

All forms of toxins are supposed to consist of two modifications, which combine in an equally energetic manner with antitoxin or with suitable substance in the cells, but differ in their resistance to heat and other destructive agents. The less resistant form passes readily into a substance called toxoid, which has the same affinity for the antitoxin as the original toxin, but is not poisonous. The facts observed, Ehrlich thinks, are best explained on the supposition that the toxic molecule contains two independent groups of atoms, one of which may be designated as the haptophorous and the other as the toxophorous group. It is by the action of the former that toxin unites with antitoxin or cell molecule and allows the latter to exert its poisonous effect. (See Figs. 67 and 70.)

The toxophorous group is unstable, but after its destruction the molecule still unites with the antitoxin or the sensitive molecule through its retained haptophorous group.

Bordet has shown that toxin unites in different multiples with antitoxin, so that the toxin molecule may have its affinity slightly, partly, or wholly satisfied by antitoxin. Slightly satisfied, it is still feebly toxic; combined with a larger amount of antitoxin, it is not toxic; but still may, when absorbed into the system, lead to the production of antitoxin. Fully saturated, it has no poisonous properties and no ability to stimulate the production of antitoxin.

**Characteristics of Exotoxins.**—Important properties of the extracellular toxins are the following: They are, so far as known, uncrystallizable, and thus differ from ptomains; they are soluble in water and they are slowly dialyzable through thin membranes, but not through thick membranes such as are used in refining antitoxic globulins; they are precipitated along with peptones by alcohol, and also by ammonium sulphate; if they are proteins they are either albumoses or allied to the albumoses; they are relatively unstable, having their toxicity diminished or destroyed by heat or freezing as well as by chemical manipulation (the degree of heat, etc., which is destructive varies much in different cases). Their potency is often altered in the precipitations practised to obtain them in a pure or concentrated condition, but among the precipitants ammonium sulphate has but moderate harmful effect. A remarkable and important characteristic of the group is that they are highly specific in their properties. They tend to attack certain groups of cells because of chemical affinities, and have the power in the infected body to excite the production of antitoxins. The diphtheria and tetanus and botulinus bacilli are the best known extracellular toxin producers.

**Precipitation of the Extracellular Toxins.**—Ammonium sulphate crystals are added to the fluid containing the toxin until it is saturated. A large excess of ammonium sulphate crystals is then added and the whole kept at about 37° C. for twelve to eighteen hours. The toxin is precipitated along with the albumoses and peptones and rises to the surface. This is skimmed off and dried in a vacuum or in an exsiccator containing strong sulphuric acid. The dried powder is placed in vacuum tubes and stored in the dark. Under these conditions the toxins deteriorate very slowly. During the process there may be a considerable loss of toxin, even when every care is taken. Tetanus toxin is especially liable to deterioration. Banzhaf has obtained a diphtheria toxin that is practically free from the meat extractions in the broth. He adds alcohol up to 65 per cent. to the slightly acidified toxin broth. The slight flocculent precipitate that is formed, after standing about one hour, is filtered off and dried in a vacuum. This dried toxin does not deteriorate. One gram of the powder contains 40,000 minimum lethal doses, whereas the original toxin broth contained 5000 minimum lethal doses per gram of solids.

**Ferment Characteristics of Toxins.**—The comparison of the action of bacteria in the tissues in the production of these toxins with what takes place in the gastric digestion has raised the question of the possibility of the elaboration by these bacteria of ferments, by which the process may be started. It would not be prudent to dogmatize as to whether the toxins do or do not belong to such an ill-defined group of substances as the ferments. It may be pointed out, however, that the essential concept of ferments is that of a body which can originate change without itself being appreciably changed, and no evidence has been adduced that toxins fulfil this condition. Another property of ferments is that, so long as the products of fermentation are removed, the action of a given amount of ferment is indefinite. In the case of toxins no evidence of such an occurrence has been found. A certain amount of a toxin is always associated with a given amount of disease effect.

**Similar Vegetable and Animal Poisons.**—Substances similar to those classed as bacterial endotoxins and as soluble toxins are formed by many varieties of cells other than bacteria. The ricin and abrin poisons obtained from the seeds of the *Ricinus communis* and the



*Abrus precatorius* have a number of properties similar to those of the diphtheria and tetanus poisons. The active poisons contained in ricin and abrin have not yet been isolated, but the impure substances are extremely poisonous. When injected into suitable animals anti-poisons are produced and accumulate in the serum. These neutralize the poisons whenever they come in contact with them.

They resemble the toxins in a general way in the manner in which they react to heat and chemicals. They are precipitated by alcohol. Through animal membranes they are less dialyzable than albumoses. Substances having these characteristics are called toxalbumins.

Poisonous snakes secrete poisons which have many of the characteristics of the bacterial albumoses. The venom contains some substances similar to peptone and others similar to globulin. The former cause general nervous symptoms and paralysis of the respiratory centre. While the latter cause intense local reaction with hemorrhages around the point of injection. The injection of venins into animals is followed by the production of antivenins which neutralize the venins. When the serum containing abundant suitable antivenins is injected into an infected person it has considerable therapeutic value.

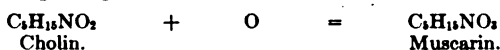
The pyogenic action of their proteins is common to all microbes, this depending principally upon their being extraneous albuminous substances. Pyogenic effects may be produced in like manner by extraneous albumins of non-microbial origin. That not every extraneous albuminous substance is harmful to the organism which seeks to resist its action is shown by those specific precipitating ferments, the precipitins, which are produced in the organisms after the introduction of every extraneous albumin.

**Ptomains.**—These were at first confused with the bacterial proteins and exotoxins. They are now known not to be found in the living tissues but they have some importance, since as products of the proteolysis caused by microorganisms, they can give rise to poisoning when ingested with putrefying foodstuffs. The ptomains are not specific, as the same ptomain may be formed by many different varieties of bacteria capable of producing protein cleavage if the nutriment and other conditions are suitable. A moderately high temperature favors their development. The matrix of these poisons is the protein nutriment. Those that are poisonous usually cause disease by being ingested preformed, but they may continue to be developed in the intestines under conditions of faulty digestion. Another method of absorption is from putrefying tissues as in gangrene.

Nencki, and later, Brieger, Vaughan and others, succeeded in isolating organic bases of a definite chemical composition out of putrefying fluids—meat, fish, old cheese, and milk—as well as from pure bacterial cultures. Some of these were found to exert poisonous effect, while others were harmless. The poisons may be present in the decomposing cadaver (hence the name ptomain, from *πτῶμα* = dead body), and, in consequence, have to be taken into consideration in questions of legal medicine. They may be formed also in the living human

body, and, if not made harmless by oxidation, may come to act therein as self-poisons or leukomains. They possess the characteristics of alkaloid bodies and have been spoken of as animal alkaloids.

Many ptomaines are known already and among them are some whose exact chemical constitution is established. Especially interesting is the substance cadaverin, which was separated by Brieger from portions of decomposing dead bodies and from cholera cultures, by reason of the fact that Ladenburg prepared it synthetically and showed it to be pentamethylenediamin  $((\text{NH}_2)_2(\text{CH}_2)_5)$ . The cholin group is particularly interesting. Cholin itself  $(\text{C}_5\text{H}_{15}\text{NO}_2)$  arises from the hydrolytic breaking-up of lecithin, the fat-like substance found in considerable amounts in the brain and other nervous tissue. By the oxidation of cholin there can be produced the highly toxic muscarin, found by Schmiedeberg in a poisonous toadstool and isolated by Brieger in certain decomposing substances:



The ptomain tyrotoxicon was obtained from cheese, milk and ice-cream by Vaughan.

Pyocyanin  $(\text{C}_{14}\text{H}_{14}\text{N}_2\text{O})$ , which produces the color of blue or blue-green pus is a ptomainic pigment. Similar bodies of a basic nature may be found in the intestinal contents as the products of bacterial decomposition. Some of these are poisons and can be absorbed into the body. Since the name ptomain was given to the poisonous products of bacterial growth before these products were chemically understood it is by many wrongly applied to all poisons found in food. Such poisoning may be due to true toxins or even living germs, such as Gärtner's bacillus or *B. botulinus*. In putrefying mixtures these products usually appear several days after the decomposition begins. If the process continues they may be changed by further cleavage to less complex non-toxic substances.

The isolation of these substances can here be only briefly referred to. According to Brieger's method, which is the one now generally employed, the cultures having a slight acid reaction (HCl) are boiled down, filtered, and the filtrate concentrated to a syrupy consistency dissolved in alcoholic (96 per cent.) solution of bichloride of mercury.

### AGGRESSINS.

Bail found that if tubercle bacilli, together with sterilized tuberculous exudate, were injected into healthy guinea-pigs, the animals died very suddenly, *i. e.*, in twenty-four hours or thereabout. The exudate alone had no appreciable effect on the animal, while inoculation with tubercle bacilli alone produced death in a number of weeks. He therefore concludes that there is something in the exudate that allows the bacilli to become more aggressive, and hence has called this hypothetical substance "aggressin." He thinks that virulent bacteria produce in the body this substance under the conditions of infection and that this aggressin

neutralizes the bacteria-destroying power of immune serum. It is an endotoxin liberated from the bacteria as a result of bacteriolysis and it acts by paralyzing the polynuclear leukocyte, thereby preventing phagocytosis. Heating the exudate to 60° C. increases rather than diminishes its aggressive properties and small doses act relatively more strongly than larger ones. These facts he explains by assuming the presence of two properties, one that prevents rapid death, is thermolabile and acts feebly in small doses, and one that favors rapid death and is thermostabile. He assumes that in a tuberculous animal the tissues are saturated with the aggressin and when fluid collects in the body cavities, as it does on injection of tubercle bacilli, it contains large quantities of aggressin, which prevent migration of the polynuclear leukocytes, but not of the lymphocytes, and hence allow the bacilli to develop freely, producing acute symptoms. In the peritoneal cavity of the normal animal injected with tubercle bacilli, on the other hand, are large numbers of polynuclear leukocytes which engulf the bacilli, thus inhibiting their rapid development, there being here no aggressin to prevent phagocytosis.

This theory has been applied to a number of infections, including typhoid, cholera, dysentery, chicken cholera, pneumonia, and staphylococcus infections. In all, similar results have been obtained as in the case of tubercle bacilli. When exudates, produced by virulent cultures of these various organisms and properly sterilized, are injected with fresh cultures into an animal, death occurs in a much shorter time than when the organisms alone are injected.

Moreover, it has been possible to immunize animals against these various infections by repeated injections of the aggressin in the form of exudates. This results in the formation of an "antiaggressin," which opposes the action of the aggressin, thereby enabling the leukocytes to take up the bacteria and thus protect the animal. This has been done in staphylococcus, dysentery, typhoid, cholera, pneumococcus, and chicken-cholera infection in animals. In addition, a very marked agglutinative property of the blood is acquired for the bacteria in the animals so immunized. Salmon and Smith, in 1884, Welch, Wassermann and others have noted somewhat similar facts. There is still uncertainty as to whether the aggressin is anything else than some of the autolyzed or split bacterial protoplasm and as to whether virulent strains produce other substances than non-virulent strains under similar conditions. If aggressins are the autolyzed bacteria, this accounts both for their aiding the virulence of the bacteria and for the production of antibodies from their injection.

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## CHAPTER IX.

### THE RESISTANCE OF THE HOST TO MICROBAL INFECTION.

THAT certain races of animals and men, and some individuals among these, are more refractory to certain diseases than are others is a fact which has long been known. Experience and observation have taught us, further, that the same individuals are at one time more resistant to disease than at another. This inborn or spontaneous refractory condition to an infectious disease is termed *natural immunity*, in contradistinction to that *acquired* by the reaction to infection. It is not always easy to distinguish between these two forms of immunity.

**Natural Immunity.**—*In Species and Races.*—In regard to variations in natural resistance between different species and races of animals, certain known facts have been accumulated. Thus cold-blooded animals are generally insusceptible to infection from those microorganisms which produce disease in warm-blooded animals, and *vice versa*. This is partly explained by the inability of the germs which grow at the temperature of warm-blooded animals to thrive at the temperature commonly existing in cold-blooded animals. But the differences observed between the several races of warm-blooded animals must have more subtle causes. The anthrax bacillus is very infectious for the mouse and guinea-pig, while the rat is not susceptible to it unless its body resistance is reduced by disease and the amount of infection is great; again, while most animals are susceptible, the common fowl is naturally immune to tetanus and rabies. The inability of the microorganism to grow in the body of an animal does not usually indicate, however, an insusceptibility to its poison; thus, for instance, rabbits are less susceptible than dogs to the effects of the poison elaborated by the pneumococci, but these bacteria develop much better in the former than in the latter.

**Individual Resistance.**—In experimental infection with minimum lethal doses inoculated in the same way a direct ratio is often noted between body weight and amount inoculated, but in spontaneous infection in both man and other higher animals individual differences in resistance are more apparent. These differences when not due to the varying number and virulence of the infecting organisms may be accounted for in part by the presence of specific antibodies in the tissues, such as the presence of diphtheria antitoxin in many human beings, and in part by other variations in the cellular activity and biochemistry of the individual.

In animals, as a whole, it is noticed experimentally that the young ones are less resistant to infection than the older and larger ones, except

in those still young enough to have immunizing substances acquired from the mother before or shortly after birth.

**Decrease of Resistance.**—The difficulty experienced by the majority of microbes in obtaining a footing and developing in the healthy body can be to a great extent removed by any cause which lowers the general or local vitality of the tissues. Among the causes which bring about such lessened resistance of the body are hunger and starvation, bad ventilation and heating, exhaustion from overexertion, exposure to cold, the deleterious effects of poisons, bacterial or other, acute or chronic diseases, vicious habits, drunkenness, etc. Purely local injuries, such as wounds, contusions, etc., give a point of entrance for infection into tissues of less resistance to invasion and which have been rendered still more vulnerable by tissue destruction. As the bacteria develop and through their poison produce adjacent injury they so predispose to further bacterial invasion in much the same way as the heat of the forest fire dries the green trees in front of it and so prepares them to ignite. Local affections, such as endocarditis, may also afford an area of lessened resistance. The presence of foreign bodies in the tissues in like manner predisposes them to bacterial invasion. Interference with free circulation of blood and retention in the body of poisonous substances which should be eliminated also tend to lessen the vitality. In these and other similar ways animals which are otherwise refractory may acquire a susceptibility to communicable disease.

**Increase of Resistance by Non-specific Means.**—All conditions which are favorable to the health of the body increase its resistance, and thus aid in preventing and overcoming infection. The internal use of antiseptics against bacteria is so far largely unsuccessful, for the reason that an amount still too small to inhibit bacterial growth is found to be poisonous to the tissue cells. The efficacy of mercury in syphilis is, possibly, an exception to the rule. In dealing with animal parasites, and with certain molds there are a number of notable exceptions to this rule, *e. g.*, quinine in malaria, emetin in amebiasis, trypan red in trypanosomiasis, and iodides in sporotrichosis. Such substances as leukocytic extract, nuclein, albumoses, and similar organic substances contained in blood serum, when introduced into the body in considerable quantity, aid to a larger extent than was formerly thought (see Vaccines) in preventing the growth of microbes. The hastening of elimination of the microbial poisons by free intestinal evacuation and encouragement of the functions of the skin and kidneys are also of some avail. The enzymes formed by certain bacteria have been found to exert a slight bactericidal action not only on the germs which have directly or indirectly produced them in the body, but also on other varieties. None of these enzymes are sufficiently protective to be of practical value, nor are they equal in power to the protective substances formed by the tissues from the bacterial products.

**Use of Local Treatment in Limiting Microbial Invasion.**—The total extirpation of the infected area by surgical means, if thoroughly

carried out, removes the microbes entirely; but, unfortunately, this procedure is rarely possible. Even when incomplete it is frequently helpful; but it may be harmful, for by creating tissue injury and exposing fresh wounded surfaces to infection it may lead to the further development of the disease. In some cases, however, like anthrax and infection from bites of rabid animals, almost complete removal of the virus, either by the knife or through cauterization, will prevent a general infection or so lessen the number of organisms in the body as to allow the germicidal element of its fluids to exterminate them. So also in tetanus, the invasion being limited, surgical interference may be of great use by removing not only the bacilli themselves, but also that portion of their poison which has not as yet been absorbed from the tissues. The beneficial effects of opening an abscess, or cleansing and draining the pleural, peritoneal or uterine cavities are well known. The retention of the poisonous products of the organisms leads to their absorption, and then through their combining with some of the tissue cells and with the protective substances of the adjacent fluids the tone of the tissues is lowered at the same time that germicidal substances have been neutralized. This enables the germs to penetrate into tissues which would otherwise resist them. The mechanical effect of pressure on the walls of an abscess by its contents also aids microbial progress and absorption of toxins. Local bleeding and the application of cold probably act by lessening absorption. The application of warmth increases the blood flow to the part, and so, when the general blood supply is germicidal, as it often is, it acts favorably on the inflammation. A similar effect of operative interference is noticed in the frequently observed beneficial result of laparotomy in tuberculous peritonitis.

Antiseptic solutions have the power of cleansing and rendering sterile the surfaces of a wound—that is, of lessening the introduction of infection. After infection has taken place, however, it is doubtful whether antiseptic washing has much more direct influence than simple cleansing, and it certainly can have no germicidal effect at any distance from the surface, either direct or indirect. Certain infectious diseases which are comparatively superficial are probably benefited by antiseptic solutions; such are gonorrhea, diphtheria, and other inflammations of the mucous membranes. Even here, however, it is impossible to do more than disinfect superficially, and in some cases any irritation of the tissues is likely to do more harm than good. In the superficial lesions of syphilis, tuberculosis, and other chronic processes, the local use of antiseptics is sometimes of great value. In these diseases the irritant effects of the antiseptics which stimulate the tissues may also be beneficial.

**The Cause of the Inability of Microbes to Grow in the Body.**—In seeking to account for the germicidal property of the blood, which to a greater or less extent affects all microbes, we cannot find it either in the insufficient or excessive concentration of the nutritive substances, or in the temperature, or in the reaction. We are thus driven to the

conclusion that the body fluids and cells contain substances which are deleterious to microorganisms.

**Acquired Immunity.**—Acquired immunity may be either active or passive. *Active immunity* is the immunity developed by the active biological response of the tissues of the host to the action of the pathogenic germs themselves or their specific products or by as yet unknown stimuli. *Passive immunity* is the immunity produced by the injection of the blood serum of animals which have previously passed through a specific disease or have been inoculated with the microbial products. This immunity is called *passive* immunity, since the animals inoculated with the immune serum take no part in the process.

**Production of Active Immunity.**—1. Through reaction to infection naturally contracted or artificially produced. According to the nature of the invading microorganisms this immunity may be slight, as after recovery from erysipelas or pneumonia, marked for a limited period of time, as in diphtheria and typhoid fever, or prolonged, as after scarlet fever or smallpox. The extent of this protection also varies in different persons after the same infection.

2. By inoculation with microorganisms attenuated by heat, chemicals, or other means. In this case an infection of the animal is produced, of moderate severity, as a rule, and the immunity is not quite as marked and lasting as after recovery from a more serious attack; but it is, nevertheless, considerable. The inoculation of sheep with the attenuated anthrax bacillus and the use of vaccination with cow-pox in man are examples of this method.

3. By the injection of the living organisms into tissues where development will not take place, as the injection of diphtheria bacilli, typhoid bacilli or cholera spirilla into the subcutaneous tissues. Here the destruction of the bacteria with the absorption of their products causes mild chemical poisoning, with considerable resulting immunity.

4. By the injection of the dead bodies of bacteria or of the chemical products which they elaborate and discharge into the surrounding culture media during their life. This produces a less marked immunity than when the living culture is used, but the method is a safer one.

5. By little understood processes by which during growth from birth to full development immunity is acquired. For instance, the majority of persons develop antitoxic immunity to diphtheria. In many cases it is probable that absorption of germs takes place from the intestines and from the respiratory tract and so provides a stimulus for the production of a small amount of antibodies. This accounts for only a small fraction of the kinds of antibodies that appear to develop during growth.

**Production of Passive Immunity.**—The first, probably, to think of the possibility of effecting this was Raynaud, who in 1877 showed that the injection of large quantities of serum derived from a vaccinated calf into an animal prevented its successful vaccination. The results obtained by v. Behring and Kitasato upon diphtheria and tetanus, where the serum

neutralizes the poisons rather than acts on the direct development of the bacteria, gave a still greater impetus to these investigations.

Suitable animals after repeated injections gradually accumulate in their blood considerable amounts of these protective substances, so that very small amounts of serum injected into another animal will inhibit the growth of the microbes or neutralize their products. Thus, 0.1 c.c. of a serum from a horse frequently injected with pneumococci will prevent the development in the body of a rabbit of many thousand times the fatal dose of very virulent pneumococci, and a few times a fatal dose of less virulent ones, the actual number as well as the virulence of the bacteria affecting the protective value of the serum.

These protective substances are found also in other fluids of the body than in the blood; they occur, indeed, in the substance of many cells to a greater or less extent (see next chapter).



FIG. 65.—Antitoxin content of 1 c.c. of the blood of two goats receiving a subcutaneous injection of 10,000 units of antitoxin at different periods of time.

The immunity produced by all of these methods affects the entire body, as is natural, since the blood and lymph which receive the protective substances from the cells or from the serum are distributed everywhere. These protective substances pass from the blood through the walls of the capillaries and finally find their way to the lymph and back to the blood. When the immunity is but slight, infection may take place in the more sensitive regions or where a large number of organisms have gained access, and still be impossible in those tissues having more natural resistance or slighter infection.

**Passive as Contrasted with Active Immunity.**—After the immune serum is injected into man the immunity is greatest at the time of its reception into the blood. This maximum, of course, is instantaneous



after an intravenous injection (Fig. 65), but is only reached after thirty-six to forty-eight hours when given subcutaneously, and then declines, being lost in several months or weeks, according as to whether or not the serum is injected into the same species of animal as the one from which it was drawn or into another species, when repeated injections are required to maintain the immunity. This passive immunity is distinctly in contrast to the active immunity acquired after the introduction of microbes or microbial products, where the tissues of the organism, in ways to us unknown, throw out, in response to the microbial stimulus, inhibitory or antitoxic substances. Here immunity may be actually lessened for one or two days (the so-called negative phase), and then, or from the beginning, it is gradually increased, and reaches its height a week or several weeks after the injection, and then slowly declines again and is lost after several months or years.

**Types of Antibodies Produced by the Inoculation of Microorganisms or Their Products.**—Antibodies may be divided into two general types: (1) those which combine with the toxins and neutralize them and (2) those which attach themselves to the microbes and together with complement or similar ferments destroy them. The nature of these substances is studied in the next chapter.

**Protective Value of Bactericidal Serums.**—The use of serums having specific protective properties has been tried practically on a large scale in man as a preventive of infection. In susceptible animals injections of some of the very virulent bacteria, as pneumococci, streptococci, meningococci, and typhoid bacilli, can be robbed of all danger if small doses of their respective serums are given before the bacteria have increased to any great extent in the body. If given later they are usually ineffective. For some bacteria, such as tubercle bacilli, no serum has been obtained of sufficient power surely to prevent infection. Through bactericidal serums, therefore, we can immunize against many infections, and even stop some just commencing; but as yet we cannot cure an infection which is already fully developed, though even here there is reason to believe that we may possibly prevent an invasion of the general system from a diseased organ, as by the pneumococcus from an infected lung in pneumonia. On the whole, the serums which simply inhibit the growth of microbes without neutralizing the toxins have not given conclusive evidence, as observed in practice, of great value in already developed disease, unless the serum can be brought in considerable concentration in contact with the infected tissues as is done by intraspinal injections in cerebrospinal meningitis.

**Relative Development of Antitoxins and Bactericidal Substances in the Different Infections.**—Although the serum of animals which have been infected with any one of many varieties of bacteria is usually both antitoxic and bactericidal, still one form of these protective substances usually greatly predominates; thus antitoxic substances are present almost exclusively in animals injected with those species of bacteria which produce powerful specific poisons—viz., the bacilli

of diphtheria and tetanus. When the toxins of either of these are injected in small amounts the animals after complete recovery are able to bear a larger dose without deleterious effects. The bacillus of symptomatic anthrax and of botulismus and the vegetable poisons ricin, croton, and abrin also produce specific antitoxins. To v. Behring and Kitasato we owe the discovery that this protecting substance accumulates to such an extent in the blood that very small amounts of serum are sufficient to protect other animals from the effects of these extracellular toxins.

There are a number of important bacteria which are believed to produce under favorable conditions a slight amount of extracellular toxin. These are the dysentery and plague bacilli, the cholera spirilla, the typhoid bacilli, the gonococci, meningococci, streptococci, etc. These latter bacteria when injected excite more of the substances which inhibit bacterial growth than of those which neutralize their toxins. So far neither protozoa nor molds have been shown to produce specific antitoxin, though with a few species agglutinins and other antibodies have been demonstrated.

The subject of therapeutic uses of serum is given after the consideration of the individual microbes (see section on Applied Microbiology, Part III).

## CHAPTER X.

### NATURE OF THE PROTECTIVE DEFENCES OF THE BODY AND THEIR MANNER OF ACTION—PHAGOCYTOSIS— EHRlich'S "SIDE-CHAIN" AND OTHER THEORIES.

BOTH the cells and the fluids of the body take part in the defense against microbial infection. The cells help in two general ways: First, by engulfing the germs and so disposing of them, thus acting as phagocytes; and second, after being acted upon by the microbes or by their products, by producing antagonistic substances that are then carried in the fluids. The fluids therefore act as passive carriers of these antagonistic bodies. All substances producing antagonistic bodies are called *antigens* and the bodies produced are called *antibodies*.

#### PHAGOCYTOSIS.

Metchnikoff, in 1883, began to publish his observations which have led to our present views on the role of phagocytosis in the protection of the body from infecting organisms. His first studies were carried on in a small crustacean in which he introduced yeast cells. He observed the struggle between the ameboid leukocytes and the yeast cells. He noted that if all the yeast cells were completely taken up by the leukocytes that the crustacean lived, otherwise it died.

He, following others, had earlier noted that dead particles of matter were engulfed by the ameba and digested. Mouton was able later to obtain a ferment from the ameba which digested albumins and even dead bacteria. Further studies made it evident that the most primitive form of digestion was carried on within the cells, while in the higher developed animals these ferments are secreted by the cells and act on materials external to them. Essentially there is no difference. Even in the flat worms it was found that the intestinal cells engulfed the suitable intestinal materials and digested them. In man it is found that numerous cells retain something of this power of engulfing and digesting foreign materials. Metchnikoff has differentiated between the motile and the fixed cells which possess these attributes. The leukocytes represent the motile while certain connective-tissue cells, endothelial cells, etc., compose the fixed cells.

Metchnikoff divides the phagocytic cells also into microphages and macrophages. The former are the polymorphonuclear leukocytes, the latter some mononuclears of the blood and the fixed tissue cells. The microphages apparently are chiefly concerned in engulfing bacteria in acute infection. In the circulating blood the polynuclear leukocytes

are the most actively motile and phagocytic elements. The small lymphocytes and mast cells almost lack phagocytic activity, although they may accumulate about an area of infection as in tuberculosis.

The action of the leukocytes in a successful resistance to infection as studied with the aid of the microscope, is briefly as follows: An emulsion of certain pyogenic cocci, for example, will be seen partly free and partly engulfed by the leukocytes. (See Fig. 66, also Figs. 109 and 111.) The phagocytosis will probably be completed in twenty-four hours. The combined action of the phagocytes and the serum will have destroyed the bacteria and at the same time those leukocytes which have engulfed too many, will themselves disintegrate. The disintegrated tissue cells, leukocytes and bacteria together with the accumulated fluids from the blood constitute, if in sufficient quantity, pus.



FIG. 66.—Streptococcus engulfed by leukocytes from abscess. Gentian-violet stain.  $\times 1000$ . (Fränkel and Pfeiffer.)

If the amount of infection is too great, either because of quantity or virulence, there will be after a period of phagocytosis an increase of the remaining bacteria. The phagocytes then disintegrate in large numbers and the bacteria more or less rapidly increase. Phagocytosis may almost cease. With the most virulent bacteria phagocytosis may be almost absent from the start.

In the chronic infections the macrophages take part as well as the microphages. When tubercle bacilli are injected into the peritoneum the leukocytes are attracted and engulf them, but they are not able to digest the waxy envelope of the bacilli, so they carry them to distant parts. Deposited in a lymph node a tubercle is formed. The macrophages here play an important part, for they probably are able to digest the bacilli.

The part played by a phagocytic cell in infection is twofold. The first is an active motion toward the infecting cell, the second is ingestion. This movement of the leukocyte is believed to be due to chemical stimuli which causes the surface tension to lessen on the part in contact. This phenomenon is called chemotaxis or tropism. As some substances repel we consider chemotaxis as positive or negative. (See also p. 59.) Massart

and Bordet showed that the products of cell destruction of leukocytes and the fixed cells of tissues as well as of bacteria possess positive chemotactic properties. This explains the gathering of leukocytes to points of tissue injury with or without bacterial infection. The presence of bacteria by their diffused proteins adds to the influence of the tissue injury.

It is still doubtful whether any bacterial products have negative chemotactic properties. Under certain conditions, especially when the bacteria are very virulent, there is no evidence of the live organisms attracting leukocytes. In the case of typhoid fever the leukopenia may be due to the action of the product of splitting the typhoid protein by the anti ferment.

**The Combined Action of Leukocytes and Serum on Microorganisms.**—The earlier observations disclosed that while anthrax and many other pathogenic bacteria were little taken up by the leukocytes in susceptible animals, nevertheless these same animals, if immunized, showed marked increase in phagocytic power. It took many investigations to clear up the reasons, but now all are agreed on the fundamental facts, that in addition to the leukocytes, serum substances, called opsonins are involved in the process of phagocytosis (see p. 217 for a detailed account of opsonins).

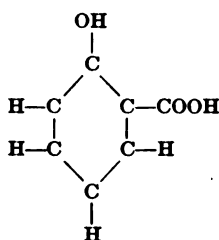
### SPECIFIC ANTIBODIES IN BLOOD SERUM.

It was early learned that the serum itself may carry substances that are antagonistic to certain germs, but it was not found out until much later that many of these substances have a specific action and can be greatly increased in power by the repeated inoculation into susceptible animals of the specific germ or its products. During this time many experimenters, chief among them Ehrlich and Bordet, studied the nature of these substances and evolved certain theories in regard to their production and interaction which have been exceedingly stimulating in promoting investigations.

**Ehrlich's Side-chain Theory.**—Ehrlich believes that the development of antibodies from the action of foreign proteins, *i. e.*, antigens, on cells is simply a reaction similar to that occurring in normal metabolism. The nutrition of cells takes place by suitable food elements entering the cells and forming physical or chemical union with their protoplasm. The cell substance must contain large numbers of various types of molecules, each of which has different atom groups. The toxins and other foreign proteins are assimilated in the same way as are food molecules and enter into a similar union. Whether or not these foreign proteins are injurious depends upon the extent of their poisonous action.

From the stand-point of organic chemistry, the living cell is conceived by Ehrlich as consisting of a vital protein nucleus and a large number of connected atom groups or "side chains" which combine with the various atom groups of nutritive materials and so nourish the cell. This is after

the analogy of the central carbon ring of any compound of the benzol series. Thus in salicylic acid the hydrogen atoms, the hydroxyl and



acid radicals represent side chains. Through these the compound can enter into relation with other substances.

Ehrlich considers the cell as an active chemical complex which is constantly eliminating waste products and through its side chains entering in new unions. Because the "side chains" are supposed to attach these new elements he names them "receptors."

Fisher has stated that a ferment to act on a substance must have an atom group which fits into some group of the fermentative substance. Ehrlich's conception of the relation of a "side chain" to a nutritive molecule is analogous.

The absorption of the antigen by the cell receptors differs from ordinary nutriment since the foreign protein is more or less poisonous and theoretically at least is believed to render useless to the cell the receptors which absorb it. The cell responds by producing new receptors and in doing so makes an excess. This is in accord with the biological law formulated by Weigert, the law of supercompensation; that is, the organism seeks to replace this defect, but in doing so, not merely replaces the receptors in question, but, according to Weigert, produces more of them than were previously present. The conditions are somewhat like those seen in the callus after a fracture, in which the organism likewise produces a greater amount of bone than was previously present.

In this way, Ehrlich states, such a large number of one type of receptors are produced by certain cells, that these become excessive; they are then thrust off into the blood, and these free receptors circulating in the blood constitute the specific antibodies. Ehrlich therefore believes that the specific antibodies in the serum are nothing more than receptors for which the substance employed in immunization possesses specific affinity. Hence the same substance which, so long as it remains a vital part of the cell, attracts the poisonous substance and enables it to exert its poisonous action on the cell, now, when it is cast off circulates free in the blood or tissue fluids, acts as a protection by intercepting, combining with and neutralizing the poison while still free in the blood, and thus prevents the poison molecule from reaching the cell itself.

The number of varieties of proteins must be enormous and theoretically also the receptors. Ehrlich has conceived that all of the antibody reactions may be separated into three orders. The first and

simplest is that to which the antitoxins belong, the second contains the agglutinins and precipitins, and the third is the most complex order, members of which require the aid of complement to complete their effect as amboceptors or cytotoxins or lysins.

The antibody reactions that are more distinctly concerned in protection from disease, namely, the antitoxin and the cytotoxin or lysin reactions, will be considered here, while the agglutinin and the precipitin reactions will be treated of in a separate chapter. The nature of antitoxin reactions, those belonging to Ehrlich's simplest order will be considered first, though historically these reactions were not observed until some time after the bactericidal (cytotoxic) qualities of serum were discovered.

### ANTITOXIN.

During the investigations on the bactericidal power of the blood the discovery of the antitoxins which combine with the toxins, but leave untouched the bacteria, was made by v. Behring and Kitasato, and the nature of the union was investigated by Ehrlich, Bordet, and others.

**Ehrlich's Theories upon Antitoxin Production.**—Ehrlich began by observing that of the many poisonous substances known to us only a comparatively small number existed against which we could truly immunize by obtaining specific antitoxic substances in the blood of the immunized organism. Let us look at two poisons which are very similar in their physiological action, for example, strychnine and tetanus poison, both of which excite spasms through the central nervous system. One, strychnine, produces no antibody whatever in the serum, while the injection of the other, the tetanus poison, causes the formation of the specific tetanus antitoxin. Ehrlich says that this is because these substances enter into entirely different relations with the cells of the living organism. The one substance, strychnine, merely enters into a loose combination with the cells of the central nervous system, so that it can again be abstracted from these cells by all kinds of solvents—*e. g.*, by shaking with ether or chloroform. The combination, therefore, is a kind of solid solution, such as has been shown in the staining with aniline dyes. The tetanus poison, on the contrary, Ehrlich says, is firmly bound to the cell; it enters the cell itself, becoming a chemical part of the same, so that it cannot again be abstracted from the cell by solvent agents. According to Ehrlich's theory the first requirement for every substance against which we can obtain a specific serum must be its power to enter into such a combination with one or more types of cells in the living animal. The substance must possess a definite chemical affinity for certain parts of the organism. Hence, in each substance against which we can specifically immunize, Ehrlich assumes a group of atoms which effects the specific binding to certain cells, the *haptophore group* (Fig. 67, B). Corresponding to this is a group in the cell of the living organism, the *receptor group*, with which the haptophore group combines. The haptophore group is distinct from that part of the substance which exerts the physiological or pathological effect in toxins,

for example, from the group which is the carrier of the poisonous action, the so-called *toxophore group* (*A*), or in ferments, from the group which

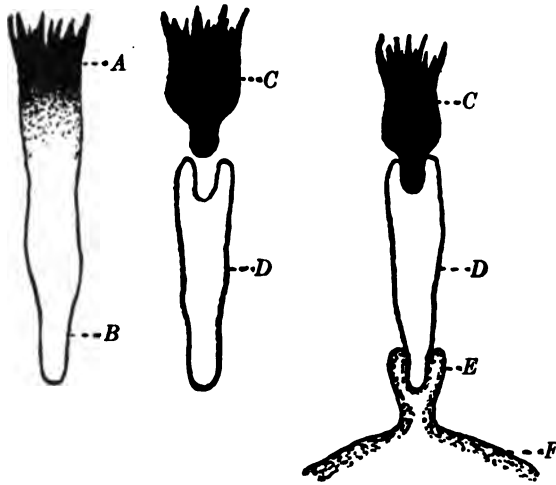


FIG. 67.—Graphic representation of receptors of the first and third orders and of complement as conceived by Ehrlich: *A*, toxophorous group of toxin; *B*, haptophorous group; *C*, complement; *D*, intermediary or immune body; *E*, foreign cell receptor; *F*, part of cell.

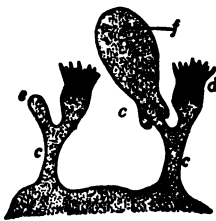


FIG. 68.—Receptors of the second order. Here *c* represents the haptophore group, and *d* the symphore group of the receptor, *f* being the food molecule with which this receptor combines. Such receptors are possessed by agglutinins and precipitins. It is to be noted that the symphore group is an integral part of the receptor.

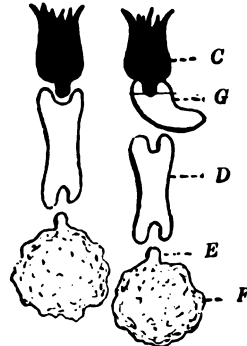


FIG. 69.—Graphic representation of amboceptors or receptors of the third order and of complement, showing on left the immune body uniting complement to foreign cell and on right the action of anticomplement, binding complement and so preventing its union with the amboceptor (see Fig. 67). *C*, complement; *D*, immune body; *E*, receptor; *F*, foreign cell; *G*, anticomplement.

exerts the ferment action, the *zymophore group*. Both groups, haptophore and zymophore, are independent of each other, and their separate



presence can easily be demonstrated because the zymophore group—*e. g.*, in poisonous toxins the toxophore group—is more readily destroyed by heat than the haptophore group. Thus by heating a toxin for some time to from 60° to 65° C. a product will be obtained which is much less poisonous, but which still possesses largely its power to bind antitoxins. In the case of toxins such substances are called *toxoids*.

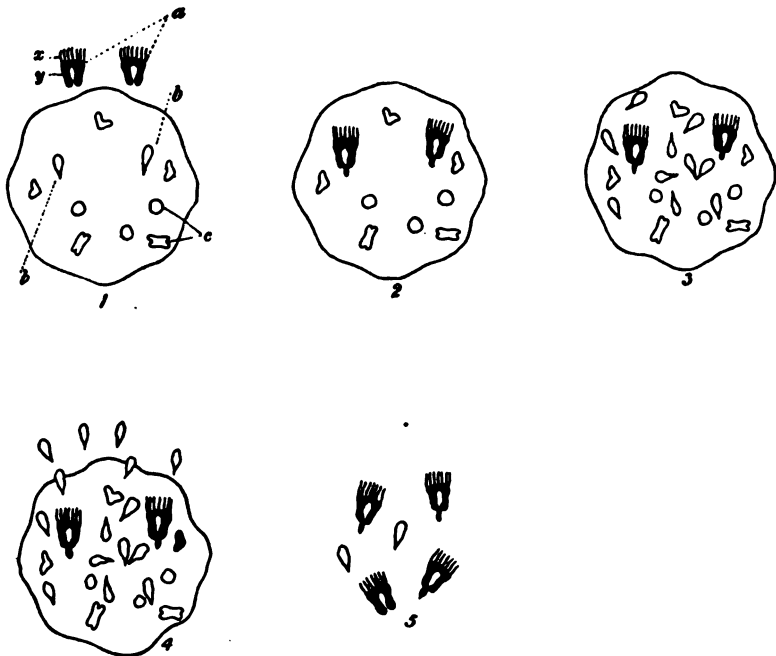


FIG. 70.—Graphic representation of Ehrlich's theory of the production of antitoxin and the neutralization of toxin: *a*, diphtheria toxin molecule; *x*, toxophore atom group; *y*, haptophore or combining group; *b*, cell receptors with affinity for diphtheria toxin; *c*, other cell receptors.

1. Cell with its receptors. Outside of cell, free toxin molecules. 2. Toxin molecules combined with the cell receptors having affinity for diphtheria toxin. 3. After three days, showing multiplication of cell receptors similar to those combined with toxin. 4. After four days, excess of receptors cast off in the blood. 5. Persons now immune. Toxin molecules entering blood neutralized by combining with free receptors in blood of immunized animal. Animal into which blood with free receptors had been transferred would possess in less quantity the same protection.

In the formation of specific antitoxins, therefore, according to Ehrlich, we must distinguish three stages (Fig. 70):

The binding of the haptophore group to the receptor (2).

The increased production of the receptors following this binding (3).

The thrusting off of these increased receptors into the blood (4).

The idea of Weigert, that the cells are biologically altered so as to continue to make receptors (antitoxin) after the cessation of the injections, and that they increase in capacity to produce antitoxin as they become accustomed to forming it through the stimulus of

repeated injections, is in partial accord with the observations made by us. The first point is opposed by the fact that there is uniformly a great drop in antitoxin ten days or two weeks after the cessation of the stimulus of the injection. The second point is, we believe, rendered improbable by the fact that by partially neutralizing toxin before injecting it into animals, we have found it possible to excite the cells to produce as much antitoxin from the first as from any later injections. Nineteen years ago we showed that an injection into a previously untreated horse of one liter of strong toxin which had been neutralized just sufficiently not to poison a guinea-pig was followed by the development of antitoxin during the following seven days so that each cubic centimeter of serum contained 60 units of antitoxin.

It is true that by the ordinary methods of immunizing the first injections of toxin produce a very small response in antitoxin, but this is because it is possible to give only minute amounts of toxin without causing the death of the animal. Very few cells are thus brought in contact with the toxin. When horses are used for a long period they cease to produce a much antitoxin as at the beginning in spite of the fact that they appear to be in good health. It does seem probable that cells which have been recently stimulated to produce antibodies and have returned to a normal condition are capable of making a considerably greater response to a later stimulus of equal intensity.

The properties of antitoxins have been most accurately studied in the case of diphtheria antitoxin. Tetanus and all other antitoxins seem to be very similar in their characteristics.

**Neutralizing Characteristics of Antitoxin.**—Diphtheria antitoxin has the power of neutralizing diphtheria toxin, so that when a certain amount is injected into an animal before or together with the toxin it overcomes its poisonous action. There is a direct action of the antitoxin upon its corresponding toxin.

The various attempts to separate the toxin and antitoxin from neutral mixtures have been failures, and it is found that neutralization takes place according to the law of multiple proportions, *i. e.*, to save an animal from 1000 fatal doses of diphtheria toxin requires little more than a hundred times as much antitoxin as is required for ten fatal doses, the resistance of the animal itself accounting for the difference.

The facts developed by the extensive studies carried out in the standardizing of antitoxin became the basis for Ehrlich's side-chain theory.

During the earlier investigations on diphtheria toxin the filtered or sterilized bouillon, in which the diphtheria bacillus had grown and produced its "toxin," was supposed to require for its neutralization an amount of antitoxin directly proportional to its toxicity as tested in guinea-pigs. Thus, if ten fatal doses of "toxin" for a guinea-pig from one specimen were required to neutralize a certain quantity of antitoxin, it was believed that ten fatal doses from every specimen, without regard to the way in which it had been produced or preserved, would also neutralize the same amount of antitoxin. Upon this belief was

founded the original v. Behring-Ehrlich definition of an antitoxin unit, viz., that it was ten times the amount of antitoxin which neutralized ten fatal doses of toxin.

The results of tests by different experimenters with the same antitoxic serum, but with different diphtheria toxins, proved this opinion to be incorrect. Ehrlich deserves the credit for first clearly perceiving this. He obtained from various sources twelve toxins and compared their neutralizing value upon antitoxin; these tests gave most interesting and important information. The results in three toxins, which are representative of the twelve, are as shown in the following table:

Toxin specimen number of Ehrlich.	Fatal doses of toxic bouillon required to kill a 250-gm. guinea-pig within 5 days, when mixed with one antitoxin unit, " $L_+$ " Ehrlich.	Fatal doses required to "completely neutralise one antitoxin unit" as determined by the health of the guinea-pig remaining unaffected " $L_+$ " Ehrlich	$L + - L_+$ = fatal doses.	Data upon "toxin" specimen given by Ehrlich.
4	39.4	33.4	6	Old, 0.003 original fatal dose, now to 0.009.
7	76.3	54.4	22	Fresh toxin taken from cultures grown more than a week and kept some days.
9	123.0	108.0	15	Fresh toxin cultures grown at 37° C. 4 days.

From the facts set forth in the table Ehrlich believed that the diphtheria bacilli in their growth produce a toxin which, so long as it remains chemically unaltered, has a definite poisonous strength with a definite value in neutralizing antitoxin. The toxin is, however, an unstable compound, and begins to change almost immediately into substances which are not, at least acutely, poisonous, but which retain at first their power to neutralize antitoxin. Later this is also lost.

The results of some experiments of Atkinson and Park were fully in accord with those published by Ehrlich as to the varying neutralizing value of a minimal fatal dose of "toxin." Moreover they also indicate roughly a general law in accordance with which these changes occur.

The neutralizing value of a fatal dose of toxin culture fluid is at its lowest in the culture fluid when the first considerable amounts of toxin have been produced, that is, at the moment of its production the toxic and neutralizing value of a toxin molecule are probably at their highest. From that time deterioration begins, but it is more rapid in the toxic portion.

While the culture is still in vigorous growth and new toxin is being produced, the neutralizing value of the fatal dose fluctuates somewhat, but with a generally upward tendency. After the cessation of toxin production the neutralizing value of the fatal dose increases steadily until it becomes five to ten times its original amount.

In our experiments the greatest value for  $L_+$  was 126, the least 27. As at six hours  $L_+$  was only 72 and at twenty-eight hours only 91, we doubt whether  $L_+$  ever reaches above 150. When we seek to analyze the above-described process we find certain facts which seem to explain it in part.

In the fluid holding the living bacilli we have, after the first few hours of toxin formation, a double process going on—one of deterioration in the toxin

<sup>1</sup>  $L_+$  = lethal doses of toxin required to kill a guinea-pig in four days after having been mixed with one unit of antitoxin.

already accumulated, which tends to increase the neutralizing value of the fatal dose; the other of new toxin formation, which probably tends to sustain the neutralizing value. Later with the period of cessation of toxin production, the gradual deterioration of the toxicity alone continues, and the fatal dose gradually and steadily increases in its neutralizing value. We believed that two or more types of toxin were produced by the bacilli.

With greater information Ehrlich was led to modify greatly the details of his earliest explanation of the reason of the variation in the ratio between toxicity and neutralizing value of toxin. He now accepts the fact that diphtheria culture fluid contains at least two toxins which differ in their characteristics.

To summarize Ehrlich's latest views as to the nature of diphtheria toxin: The diphtheria bacillus secretes two toxic substances, one of which, the toxin, causes the acute phenomena of diphtheria intoxication, while the other, the toxon, causes cachexia and paralysis after a rather long period of incubation. The less toxic toxin, or toxoid, appears as the result of the degeneration of the toxophore group of the toxin, the haptophore group remaining intact. The toxin may be separated into three divisions, which vary in their affinity for antitoxin—prototoxin, deuterotoxin, and tritotoxin. On the same basis there are three toxoids—prototoxoids, syntoxoids, and epitoxoid (the toxon)—the first of which has the greatest affinity for antitoxin, while the epitoxoid has the least. The toxins are divided into an alpha and a beta portion, depending on the ease with which they are changed into toxoids. All of these substances unite with tissue cells and with antitoxin through the agency of a haptophore group, while the toxicity depends on the presence of a toxophore group in the toxin or toxon molecule.

Bordet and others refuse to accept these conceptions of Ehrlich and the whole matter is at the present time still under discussion. Thus the existence or non-existence of toxons has excited a great deal of discussion among investigators. The Swedish chemists, Arrhenius and Madsen, have given much attention to toxons and are applying the principles of physical chemistry to the study of toxons and antitoxins. It is a well-known fact that some chemical substances when in solution have the power of breaking up into their constituent parts; thus sodium chloride breaks up in part into sodium and chlorine, as sodium or chlorine ions or electrolytes. The dissociated sodium and chlorine may then enter into combination with any other suitable substance which may be present. Arrhenius holds that this is the case with the toxin-antitoxin molecule, that it may to a certain extent again break up into separate toxin and antitoxin. He believes that this dissociated toxin is the substance which Ehrlich has been calling toxon. Madsen, who formerly had done much work with toxons, has now joined with Arrhenius in support of the dissociation theory. In spite of their reasoning Ehrlich and his followers continue to uphold the toxon as an independent toxic substance. Recent investigations throw doubt on both explanations as being at all final.

**Nature of Diphtheria and Tetanus Antitoxin.**—Experiments have seemed to show that it was either closely bound to the serum globulins or was itself a substance of protein nature closely allied to serum globulin. A fact developed by Atkinson is that the globulins tend to increase markedly in the serum of horses as the antitoxic strength increases. It seems possible from the above that diphtheria antitoxin has the characteristics of the serum globulins. Antitoxin is only little injured by prolonged moderate heat (56° C.), but is destroyed by short exposure to higher temperatures (95° to 100° C.). It is less sensitive than true toxin. Atkinson, when research chemist in our laboratory, found that in the case of antitoxic serum the globulin precipitate carries with it all of the antitoxic power of the serum, leaving the filtrate without

any neutralizing power against the diphtheria toxin. Independently of Atkinson, Pick obtained similar results. These experiments were continued later by Gibson and Banzhaf, who proved that the globulins of the horse which were insoluble in saturated sodium chloride solution carried with them no antitoxin. The soluble globulins which become insoluble on heating also contain no antitoxin. With this knowledge a practical method of eliminating much of the non-antitoxic portion of the serum was perfected.

**Production of Antitoxin for Therapeutic Purposes. — Diphtheria Antitoxin.**—As a result of the work of years in the laboratories of the Health Department of New York City, the following may be laid down as a practical method:

A strong diphtheria or tetanus toxin should be obtained by taking a very virulent culture and growing it in broth under the conditions already described. *The following directions apply to the production of diphtheria antitoxin:*

The horses used should be young, vigorous, of fair size, and absolutely healthy. The horses are severally injected with 10,000 units of antitoxin so as to allow giving a much larger dose of toxin<sup>1</sup> than would otherwise be safe and thus gain several weeks in time. The following figures give the actual injections in a horse which produced an unusually high grade of serum:

An injection was first given of 10,000 units of diphtheria antitoxin.

Injections of toxin were given at first every two days and then later every three days in the following amounts.

First day, 12 c.c. toxin (0.0025 c.c. fatal dose).

Second and later injections of toxin without antitoxin at three-day intervals as follows: 15 c.c., 20 c.c., 30 c.c., 40 c.c., 50 c.c., 60 c.c., 80 c.c., 100 c.c., 125 c.c., 150 c.c., 170 c.c., 205 c.c., 250 c.c., 300 c.c. (fortieth day). The injections were gradually increased until, on the sixtieth day, 675 c.c. were given. The whole injection should not be in one place, but divided into six or eight portions.

The antitoxic strength of the serum was on the twenty-eighth day, 225 units; on the fortieth day, 850 units; and on the sixtieth day, 1000 units. Regular bleedings were made weekly for the next four months, when the serum had fallen to 600 units in spite of weekly, gradually increasing doses of toxin. If the antitoxin is not given we begin with 10 fatal (for guinea-pigs) doses of toxin diluted to 10 c.c. and increase the amount each time about 25 per cent.

There is absolutely no way of judging which horses will produce the highest grades of antitoxin. Very roughly, those horses which are extremely sensitive and those which react hardly at all are the poorest, but even here there are exceptions. The only way, therefore, is to bleed the horses and test their serum at the end of six weeks or two months. If only high-grade serum is wanted

<sup>1</sup> The culture, after a week's growth, is removed, and having been tested for purity by microscopic and culture tests is rendered sterile by the addition of 10 per cent. of a 5 per cent. solution of carbolic acid. After forty-eight hours the dead bacilli have settled on the bottom of the jar and the clear fluid above is siphoned off, filtered, and stored in full bottles in a cold place until needed. Its strength is then tested by giving a series of guinea-pigs carefully measured amounts. Less than 0.005 c.c., when injected hypodermically, should kill a 250-gram guinea-pig.

all horses that give less than 150 units per cubic centimeter are discarded. The retained horses receive steadily increasing doses, the rapidity of the increase and the interval of time between the doses (two days to one week) depending somewhat on the reaction following the injection, an elevation of temperature of more than 3° F. being undesirable. At the end of three months the antitoxin serum of all the horses should contain over 300 units, and in about 10 per cent. as much as 800 units in each cubic centimeter. Not more than 1 per cent. give above 1000 units, and none so far has given as much as 2000 units per cubic centimeter. The very best horses if pushed to their limit continue to furnish blood containing the maximum amount of antitoxin for several months, and then, in spite of increasing injections of toxin, begin to furnish blood of gradually decreasing strength. If an interval of three months' freedom from inoculations is given every nine months, the best horses furnish high-grade serum during their periods of treatment for from two to four years.

**Tetanus Antitoxin.**—This is carried on exactly as in the case of diphtheria antitoxin except that one proceeds more slowly. Good horses yield a serum containing 200 to 600 units per cubic centimeter.

**The Handling of the Antitoxic Serum or Plasma.**—For the collection and preservation of the antitoxin the blood is withdrawn from the jugular vein by means of a sharp-pointed cannula, which is plunged through the vein wall, a slit having been made in the skin. The blood is carried by a sterile rubber tube into large Erlenmeyer flasks, held slanted, or into cylindrical jars, and allowed to clot. The serum is drawn off after four days by means of sterile glass and rubber tubing, and is stored in large flasks. When the globulins are to be separated the blood may be added directly to one-tenth of its volume of a 10 per cent. solution of sodium citrate. This prevents clotting of the blood. Small phials are filled with the serum or globulin solution. The phials and their stoppers, as indeed all the utensils used for holding the serum, must be absolutely sterile, and every possible precaution must be taken to avoid contamination. An antiseptic may be added to the serum as a preservative, but it is not necessary except when the serum is to be sent to great distances, where it cannot be kept under supervision.

Kept from access of air and light and in a cold place it is fairly stable, deteriorating not more than 30 per cent., and often much less within a year. Diphtheria antitoxin, when stored in phials and kept under the above conditions, contains within 10 per cent. of its original strength for at least two months; after that it can be used by allowing for a maximum deterioration of 2 per cent. for each month. The antitoxin in old serum is just as effective as in that freshly bottled, only there is less of it. The serum itself is less apt to produce rashes. All producers put more units in the phials than the label calls for, so as to allow for gradual loss of strength.

**Standardizing of Antitoxin Testing.**—Ehrlich, with the knowledge gained by his investigations, called attention to the necessity of all laboratories using the same toxin so that a unit would become a definite amount. The German Government undertook to supply this need by testing all sera produced in Germany and by supplying a carefully tested serum to be used by the producers as a standard against which to test their toxins and antitoxins. In this way smaller testing stations can

make their results correspond with those of the central station. The United States Marine Hospital Laboratory has also distributed to laboratories in the United States an equally carefully standardized serum for diphtheria and a toxin for tetanus.

It is seen that the old definition of v. Behring and Ehrlich, that a *diphtheria antitoxin unit* contains the amount of antitoxin which will protect the life of a guinea-pig from one hundred fatal doses of toxin, is true only in a general sense and for freshly prepared toxins. The important point to remember is that for fourteen years the German, United States, and other governments have kept carefully preserved a serum which is supplied to all testing stations, so that the unit measure is the same in all countries and from year to year.

The actual test to estimate the number of units in 1 c.c. of an antitoxin serum is therefore carried out as follows: Six guinea-pigs are injected with mixtures of tested toxin and the serum to be tested. In each of the mixtures there is the amount of toxin sufficient to just neutralize 1 unit of antitoxin. For purposes of testing, in order to avoid delay and secure definiteness, the toxin is considered exactly neutralized when the guinea-pig remains alive for four days and dies on the fifth. In each of the mixtures the amount of the unknown serum varies; for instance, No. 1 would contain 0.002 c.c. serum; No. 2, 0.003 c.c.; No. 3, 0.004 c.c.; No. 4, 0.005 c.c., etc. If at the end of four days the guinea-pigs, Nos. 1, 2 and 3 were dead and Nos. 4, 5 and 6 were alive we would consider the serum to contain over 200 units per cubic centimeter, and less than 250 units because 0.004 ( $\frac{1}{250}$ ) c.c. of the serum did not protect the guinea-pig for a full four days, while  $\frac{1}{200}$  c.c. protected the guinea-pig for longer than four days. We would then test again between these limits. When we test for experimental purposes sera with very little antitoxin, we often use only one-tenth the above amount of toxin and so estimate the quantity of serum holding one-tenth of a unit.

The mixed toxin and antitoxin must remain together for at least fifteen minutes before injecting, so that complete union may occur.

*Intracutaneous Test.*—Doerr showed that very small amounts of the mixture could be injected intracutaneously, and non-neutralized toxin give a clear-cut local lesion.

*Testing Tetanus Antitoxin.*—Tetanus antitoxin is tested in the same way except that the serum is tested against 1000 fatal doses of standard tetanus toxin instead of 100 fatal doses as in diphtheria toxin.

**The Separation and Concentration of Antitoxin.**—There have already been many attempts to accomplish this in the case of the antitoxins. Those interested in the chemical side of these investigations are referred to the article by Banzhaf, given in References. Gibson, as we have already stated, by placing the ammonium sulphate precipitate from the antitoxic serum in saturated sodium chloride solution, found that the portion of the globulin soluble in this contained all the antitoxin. In this way the nucleoproteins and the insoluble globulins present in the Atkinson preparation were eliminated, as the following summary shows:

Ordinary antitoxic serum contains serum globulins (antitoxic), serum globulins (non-antitoxic), serum albumins (non-antitoxic), serum nucleoproteins (non-antitoxic), cholesterin, lecithin, traces of bile-coloring matter, traces of bile salts and acids, traces of inorganic blood salts and other non-protein compounds. Refined serum contains serum globulins (antitoxic), traces of serum globulins (non-antitoxic), dissolved in dilute saline solution. Later, Dr. E. J. Banzhaf, who had succeeded Gibson, discovered that if the antitoxic serum or plasma was heated to 57° for eighteen hours there was a change of a considerable portion of the soluble globulins into insoluble globulins. The antitoxin remained unchanged. This permitted a greater elimination of the non-antitoxic proteins.

**METHOD OF CONCENTRATION.**—The material we use is blood plasma instead of blood serum. This is obtained by allowing the blood to flow directly from the jugular vein of the immunized horse into 10 per cent. sodium citrate solution, which prevents it from clotting and allows the red corpuscles to settle out. This plasma is used, in place of serum, merely as a matter of convenience and economy.

**ISOLATING THE ANTITOXIN GLOBULINS**—From year to year Banzhaf has succeeded in discovering methods for further purifying the antitoxin. These methods have greatly lessened the early rashes and subsequent "serum sickness." Briefly his latest method is as follows: The citrated plasma is diluted with half its volume of water and saturated ammonium sulphate solution is added up to 30 per cent. saturated solution. This mixture is heated up to 60° C. and held there for one hour. Then filtered while hot. The precipitate contains the native non-antitoxic proteins and a large amount of non-antitoxic proteins newly formed by the above method of heating. This precipitate is discarded. The filtrate is brought up to 50 per cent. saturated ammonium sulphate solution. The resulting precipitate contains only pseudoglobulin and antitoxin. The albumin remains in solutions and is discarded. The pseudoglobulins and antitoxin precipitate are pressed to remove excess of fluid and then dialyzed until free from salts. After dialysis is complete 0.8 per cent. sodium chloride is added for isotonicity and 0.3 per cent. trikresol for preservation. It is then filtered through paper pulp to remove extraneous matter, then through a Berkefeld clay filter to remove bacteria, tested for sterility and potency and filled in sterile syringes. The above method gives a concentration of about six times the original potency.

### CYTOTOXINS OR CYTOLYSINS.

The bactericidal effect upon bacteria of the blood serum, noted by Nuttall in 1886, is now undisputed, and is readily shown by the fact that moderate numbers of bacteria when inoculated into the blood stream usually die soon, and this destruction may be so rapid that in a few hours none of millions remains alive. Even when some of the bacteria survive there is for a time a decrease in the number living. According to their virulence the survivors more or less rapidly increase. Toward the most virulent microorganisms the serum of a man or animal which has not been immunized by a previous infection may afford almost no antagonism. Buchner, in 1889, showed that serum heated to 55° lost its destructive power. He believed that in serum there was but a single bactericidal substance and called it alexin.



In 1894-95 Pfeiffer showed that when some fresh culture of the typhoid bacillus on agar is added to serum from an animal immunized against typhoid bacilli and the mixture injected into the peritoneal cavity of a non-immunized guinea-pig the following phenomenon is observed. If from time to time minute drops of the liquid be withdrawn in a capillary tube and examined microscopically, it is found that the bacteria previously motile and vigorous, and which remain so in control animals inoculated without the specific serum, rapidly lose their motility and die. They are first immobilized, then they become somewhat swollen and agglomerated into balls or clumps, which gradually become paler and paler, until finally they are dissolved in the peritoneal fluid. This process (lysis) takes place in about twenty minutes, provided a sufficient degree of immunity was present in the animals from which the serum was obtained. The animals injected with the mixture of the serum of immunized animals and typhoid cultures remain unaffected, while control animals treated with a fluid containing only the serum of non-immunized animals mixed with typhoid cultures die. Pfeiffer claimed that the reaction of the serum thus employed is so distinctly specific that it can serve for the differential diagnosis of the cholera vibrio or typhoid bacillus from other vibrios or allied bacilli, such as Finkler's and Prior's or those of the colon group respectively. He thus showed that there is a great increase in the bactericidal power of a serum after immunization for the species of bacteria used in immunization. Metchnikoff then showed that the immunized serum added to peritoneal fluid in the test-tube has the same bactericidal or lytic effect on the spirilla.

Bordet, in 1895, reported that defibrinated blood filtered free of blood cells should be used instead of the peritoneal fluid and that if to a serum from an immunized animal, which had lost its bactericidal power through age, fresh serum from an untreated animal was added, the serum regained its destructive powers, *i. e.*, it was activated, although the fresh serum by itself had almost no effect. These observations of Pfeiffer and Bordet indicated clearly that two types of substances at least were required to destroy cells. Both of these were present in fresh immune serum, one of which was stable and more or less specific, and the other unstable and non-specific. The latter was proven to be present in all blood, while the former existed, except in minute amount, only in the blood of the immunized or in that of those naturally immune. The number of microbes introduced in a germicidal test is of great importance; for the serum with its contained substances is capable of destroying only a certain number, and after that it has lost its germicidal properties.

Thus the following test illustrates this:

No. of bacteria in 1 c.c. fluid	Amount of serum added	Approximate number alive after being kept at 37° C.		
		One hour	Two hours	Four hours
30,000	0.1 c.c.	400	2	0
100,000	0.1 c.c.	5,000	1,000	2,000
1,000,000	0.1 c.c.	400,000	1,000,000	5,000,000

Haas found that the circulating blood is not always bactericidal for any given variety of bacteria to the same extent the serum is. The reasons for this are not entirely clear, but it is believed that the leukocytes give up ferments to the serum as they break down.

**Names Attached to Substances Producing Cytolysis.**—Different investigators have applied to them different names. The one which is resistant to heat, which attaches itself directly to the cells, even at low temperatures, and is increased during immunization, is called sensitizing substance, interbody, amboceptor, or immune body. The other, which is sensitive to heat, is present in the healthy normal serum, is not increased during immunization, and unites with the cells' protoplasm only at temperatures above the freezing-point, is called alexin, or complement.

The immune body attaches itself to the cellular substance, but does not appreciably harm the cells. The complement destroys the cells after the immune body has made the cell vulnerable.

According to Ehrlich the specific immune body first unites with the protoplasm of the foreign microbe or cell and this develops in the immune body an affinity for the complement and the two unite. (See Fig. 67.) He believes that it is through the immune body that the complement exerts its action on the cell. Very similar to the immune body is the substance called opsonin. This unites with the cell, but instead of making it sensitive to the complement it makes it sensitive to some ferment contained in the leukocytes. (See chapter on Opsonins.)

**The Relation of Bacteriolytic to Hemolytic Serums.**—Bordet, through his own researches and those of Gruber and Durham, was able to show that the same type of reaction took place in the animal body when cells of any kind were injected. He showed, for instance, that there was a close similarity between bacteria and the cells of the blood. By immunizing an animal, species *A*, with red blood cells of animal, species *B*, he found that the blood of *A* became hemolytic for the cells of *B*, just as if immunized with cholera spirilla it would have been bacteriolytic for cholera spirilla. Since then truths obtained from investigation with any type of cells have been applied equally to all others. This made it possible for Ehrlich, Bordet, and others to study the nature of these processes upon red blood cells instead of bacteria.

**Experiments Devised by Ehrlich to Show the Nature of Cytolytic (Bacteriolytic, Hemolytic, etc.) Substances in the Blood.**—Ehrlich asked himself two questions: (1) What relation does the hemolytic serum or its two active components, immune body and complement, bear to the cell to be dissolved? (2) On what does the specificity of this hemolytic process depend? He made his experiments with a hemolytic serum that had been derived from a goat treated with red cells of a sheep. This serum, therefore, was hemolytic specifically for sheep-blood cells, i. e., it possessed increased solvent properties exclusively for sheep-blood cells. Ehrlich argued as follows: "If the hemolysin is able to exert a specific solvent action on sheep-blood cells, then either of its two factors the immune body or the alexin (complement) of normal serum, must possess a specific affinity for these red cells." To show this he devised in conjunction with Morgenroth the following series of experiments:

**EXPERIMENT 1.**—The serum that was specifically hemolytic for sheep-blood cells was made inactive by heating to  $55^{\circ}\text{C}$ ., so that then it contained only the heat resistant substance (immune body). To this was then added a sufficient quantity of sheep red blood cells, and after a time the mixture was centrifuged. Ehrlich and Morgenroth were now able to show that the red cells had combined with all the heat-resistant substances, and that the supernatant clear liquid was free from the same. In order to prove that such was the case they proceeded thus: To some of the clear centrifuged fluid they added more sheep red cells; and, in order to reactivate the serum, a sufficient amount of alexin in the form of normal serum was also added. The cells, however, did not dissolve since there was no sensitizing substance. The next point to prove was that immune body had actually combined with red cells. The red cells which had been separated by the centrifuge were mixed with a little normal salt solution after freeing them as much as possible from fluid. Then a little alexin in the form of normal serum was added. After remaining thus for two hours at  $37^{\circ}\text{C}$ . these cells had all given up their hemoglobin to the surrounding fluid.

In this experiment, therefore, the red cells had combined with all the sensitizing substance, entirely freeing the serum of the same.

The second important question solved by these authors was this: What relation does the alexin bear to the red cells? They studied this by means of a series of experiments similar to the preceding.

**EXPERIMENT 2.**—Sheep red blood cells were mixed with normal, *i. e.*, *not hemolytic* goat serum. After a time the mixture was centrifuged and the washed red cells tested by the addition of sensitizing substance to determine the presence of alexin. It was found that in this case the red cells, in direct contrast to their behavior toward the sensitizing substance in the first experiment, did not combine with even the smallest portion of alexin, and remained unchanged. This experiment showed that the sensitizing substance first combined with the cell and then only could the alexin unite with the combined cell-immune body complex.

**EXPERIMENT 3.**—The third series of experiments was undertaken to show what relations existed between the blood cells on the one hand and the sensitizing substance and the alexin on the other, when both were present at the same time, and not, as in the other experiments, when they were present separately. This investigation was complicated by the fact that the specific immune serum very rapidly dissolves the red cells for which it is specific, and that any prolonged contact between the cells and the serum at ordinary temperatures, in order to effect union, is out of the question. Ehrlich and Morgenroth found that at  $0^{\circ}\text{C}$ . no solution of the red cells by the hemolytic serum takes place. They therefore mixed some of their specific hemolytic serum with sheep-blood cells, and kept this mixture at  $0^{\circ}$  to  $3^{\circ}\text{C}$ . for several hours. No solution took place. They now centrifuged and tested both the sedimented red cells and the clear supernatant serum. It was found that at the temperature  $0^{\circ}$  to  $3^{\circ}\text{C}$ . the red cells had combined with all of the sensitizing substance, but had left the alexin practically untouched.

The addition of red cells in the experiments was always in the form of a 5 per cent. mixture or suspension in 0.85 per cent., *i. e.*, isotonic salt solution.

The significance of the last of the above-cited experiments is, according to Ehrlich, at once apparent. It is that the sensitizing substance possesses one combining group with an intense affinity (active even at  $0^{\circ}\text{C}$ .) for the red cell, and a second group possessing a weaker affinity (one requiring a higher temperature) for the alexin.

**Bordet's Theory.**—Bordet supposes that, instead of the tissue cells, whose receptors have combined with the toxin or foreign cell substance (antigen, haptine), producing an excess of similar receptors, they build up substances which in their character are not identical with pre-existent principles. These new substances have become endowed with

a more marked affinity for the specific antigen in question. Bordet considers that Ehrlich, in offering explanations which seem definitive, has caused certain problems which have scarcely been touched upon to be regarded as settled. According to Bordet, Ehrlich is wrong in attributing such special properties to the immune body alone rather than to both antigen and immune body to an equal degree. He states that, "as a matter of fact, these phenomena should be related, not as regards antigen or antibody considered separately, but as regards the complexes which result from their union, and it is evident that the special properties of the antigen must affect markedly and perhaps to a preponderating degree the qualities of such complexes. Just as the union of agglutinins with microbes produces in them a remarkable sensitivity to the agglutinating effect of electrolytes by modifying their property of molecular adhesion, in a similar way sensitizers confer on their antigens a similar modified property of adhesions, namely, alexin absorption." In his opinion, antibodies, whatever their nature, act very much alike; but the effects which they produce differ with the antigen in question.

Muir has shown that when cells are saturated with both immune body and complement, the addition of fresh cells causes a splitting off of immune body, but not of complement. This throws further doubt upon the supposed direct union of immune body and complement.

There are exceptional normal sera, the complement of which may be fixed by certain cells without the presence of an immune serum. Malvoz showed that this is the case with dog serum mixed with *B. anthracis*. This serum acts, moreover, as if it contained a true sensitizer, because, in the presence of this organism it will cause the fixation of the complement of the sera of rabbits and guinea-pigs.

Most of the experiments which have been made with the purpose of clearing up these difficult problems have been made upon red blood cells. Here the absorption of the immune bodies at low temperatures and the lack of noticeable injury until the complement is added at a suitably high temperature is very striking.

**Multiplicity of Immune Bodies and Complements.**—The immune bodies are very numerous and fairly specific in their action. The complement substance is much less specific and, though probably multiple, when chemically considered, each variety acts upon widely different bacteria and cells after they have united with the immune body. There is little reason to think that the complement of one animal is any more capable of attacking cells prepared by immune bodies developed in its blood than by immune bodies developed in some other species.

**Antiamboceptors and Anticomplementary Substances.**—Antiamboceptors have been produced by Bordet, Ehrlich and others against hemolytic amboceptors by graded inoculations with hemolytic serums. Anticomplements (Fig. 69) have also been produced. There are, naturally, many non-specific substances in the blood, such as certain lipoids and globulins that inhibit complement action. Some of these anticomplementary substances may be destroyed by a low degree of heat, that is, by inactivation of serum.

**Relation between Virulence and the Building of Immune Bodies.—**

It is believed by most to take place the more rapidly the more virulent the infecting organisms. In our experiments this has not been evident with all kinds of bacteria. It must be remembered that increase of virulence for one species of animal does not mean increase for all animals; so that in order to draw conclusions, the animal upon which the virulence is tested must be the same variety as the one being immunized.

**Origin of Immune Bodies.**—Their source must undoubtedly be attributed to the cells, but probably only certain cells produce them, chiefly certain cells in the blood-forming organs, such as the bone marrow, spleen, and lymph nodes. The red blood cells seem rather to destroy than to increase them. Injections of toxins and microbial substances into the lung and into the subcutaneous tissues give rise to the formation of antibodies which are certainly formed partly, if not wholly, locally, and later find their way to the blood. The nuclein derived from the cells, though it has a general germicidal action, and may enter into the complement (alexin), has different properties, and so cannot itself be one of these bodies.

**Origin of Complement (Alexin).**—The cells which have abundant nuclear substance, such as the leukocytes and lymph cells, seem especially to be a source, and Metchnikoff asserts their preëminent role as the producers of both complements and immune bodies. Buchner and others have found that after injection of bacterial filtrates the leukocytes were attracted in great numbers to the region of injection, and that the fluid there, which was rich in leukocytes, was more bactericidal than that of the blood serum elsewhere. Some claim to have demonstrated that along with increased leukocytosis there is a general increase in the complement in the blood; still it has not yet been positively established that the complement is derived solely from the leukocytes, nor from all leukocytes, and a mere increase in them does not always mean an increase in the complement.

**Multipartial or Polyvalent Sera.**—Microbes are not homogeneous masses of protoplasm, but are made up of various molecules which differ biologically from one another. Conforming to this, the anti-substances, immune bodies (antitoxins, opsonins, etc.), which appear in a serum are made up of the sum of the antibodies which correspond to these different protein substances in the microorganisms. An immune serum of a person recovered from an infection therefore consists of the partial groups which correspond to the separate partial elements of the microbial body which produced the infection. We are further able to show that these partial elements in one and the same microbial species are not the same for all individuals of that species. Thus one culture of streptococci or of *Bacillus coli* may have a few partial elements which differ from those of a culture of another strain. What is the consequence of this? The consequence will be that when we immunize with a culture *a* of such bacteria we shall obtain a serum which acts completely on cultures of this organism or on cultures of a similar strain, for in this serum all the partial elements present in culture *a* are represented.

If, however, we employ culture *b*, *c*, or *d*, which perhaps possesses other partial elements, we shall find that the serum does not completely affect each culture separately. As already stated, such a condition of things is found in inflammations due to streptococci and other bacteria, and is therefore of considerable practical importance. It is because of this fact that a serum from an animal immunized to one culture acts best only in a certain percentage of cases. In order to overcome this difficulty in persons infected with these bacterial species we have no choice but to make serums, not by means of *one* culture, but by means of a number of different strains of the same species. The difficulty of early identification of the strain and of having sufficient animals to provide a separate serum for each strain makes this necessary. The result of this will be that, corresponding to the various partial elements in these different cultures, we shall obtain a serum containing a large number of the partial groups. Such a serum will then exert a specific action on a large number of different cultures, but not quite as great an influence on any one as if only that variety had been injected.

Owing to these partial groups also a serum—*e. g.*, antityphoid serum—can specifically affect to a very slight degree a closely allied species of bacterium, such as the paratyphoid and to a slight extent an even more distantly connected species, like *Bacillus coli*. For it is known that related species of bacteria possess certain partial groups in common, and a serum is thus produced which to a certain extent acts on such allied species. This constitutes what is known as “group action.”

**General Methods Used in Production of Antibacterial Sera.**—*Immunization of Horses.*—Subcutaneous injections have been practised in the past, but this method has certain objections which have led to the almost general adoption of intravenous inoculation. With the latter method, a high degree of immunity is more quickly established, the amount of culture required less, and certain complications avoided, such as the development of abscesses after meningococcus vaccines. The frequency of inoculation will depend on conditions. Daily intravenous injections alternating with periods of rest is the most rapid method of producing antisera. Flexner has applied this method, giving 3 successive daily injections followed by a week's rest. We have found injections once a week adequate in maintaining the titre of the sera after the maximum is reached.

*Preparation of Vaccine.*—This varies with the bacterium employed. The easily growing types are cultivated on agar and suspended in saline. Meningococci are grown on glucose agar. Streptococci may be grown on agar or in broth and treated as are pneumococci. The last is grown in broth, the cocci thrown down and then washed with saline solution and finally suspended in saline for injection. Injection of the broth or culture ingredients is to be avoided as serious reactions or even sudden death may occur. This has been attributed to sensitization to the peptone-like substances. The relation of virulence to antibody production is not well understood. It is usually advised that the pneumococci be kept fully virulent for mice. Stock strains are used for the other species of microorganisms.

*Selection of Strains.*—This will depend on whether a univalent, polyvalent or a serum against representative strains is desired. In the case of the meningococci the strains should cover all agglutinative variants. With pneumococci, a serum against both Types I and II can be produced with antibody content against each as active as in a univalent serum. With streptococci, the representative hemolytic strains are employed as we have little reason to include the viridans type as they are practically heterogeneous and even sera from

autogenous strains have given no results in chronic endocarditis. Torrey's ten strains of gonococci are usually employed. For dysentery sera representative types are employed.

*Horses.*—Healthy horses are selected. (Deformities or crippling is no bar.) They are tested for glanders with mallein by the complement-fixation reaction. An injection of tetanus antitoxin is given every few months.

*Dosage.*—The dosage depends on the toxicity of the strains employed and the temperature and general reaction of the horse as well as his general health. Specific dosage, therefore, cannot be given. Depending on the type of bacterium  $\frac{1}{20}$  to  $\frac{1}{10}$  of an agar slant or 1 to 2 c.c. of a broth culture is the initial intravenous dose. This is then gradually increased until a maximum is reached. When injections are given only once a week, larger doses may be given than when given daily. After the injections, temperatures should be taken frequently. The optimum dose causes a sharp febrile reaction to about 104° F., but the temperature returns to normal in eight to twelve hours. A chill may occur. The maximum dose is the amount that will maintain the required antibody content and the balance of antibodies if several strains are employed, but if this requires doses which give excessive reactions, the horses' period of productivity will be short. Desensitization as advised by Dopter and Amoss and Wollstein, that is, the preliminary inoculation of a small dose, has been of no value in our hands. With most bacteria live cultures may be used from the start. Streptococci and pneumococci had better be killed at first. Autolysates (salt solution suspensions covered with toluol and stored on ice) of meningococci were advised at first by Flexner. Amoss and Wollstein found that the autolysate alone gives rise to serum of poor quality. This is contrary to our experience. We maintained the antibody content of a horse for a period of over six months with the injection of autolysate alone. More observations are needed: its advantage is its convenience, its disadvantage the relatively greater reactions it produces in the horses so that the dose should be smaller than its equivalent in live cocci.

*Titration of Sera. Standards.*—The methods employed and the standards adopted, vary to a great extent, as the problem involved is much more complex than with toxin-antitoxin methods. The methods possible of application are dilution titration of the antibodies, agglutinins, opsonins and bactericidal content and the determination of the protective value when injected with cultures. The agglutinin content is chiefly of value with typhoid and dysentery, although the content in these antibodies may decline after prolonged inoculation without, as far as we know, a coincident fall of other antibodies. The agglutination reaction is especially of value in determining the probable antibody balance where several or many types of strains are employed as with dysentery and meningococci, this being the guide as to the pro-rata amounts to be injected into the horses. Opsonic determination is only of comparative value. It cannot be used as a basis for a standard as it is open to serious errors and variations beyond control. The bactericidal titration has found only limited application. The complement-fixation reaction has been of value in titrating meningococcus serum, and a rough standard that not more than 0.002 c.c. shall be required to give complete fixation, using a mixed antigen, is employed in several laboratories. The protective value of meningococcus serum, using white mice (Hitchens and Robinson), promises to be of value. For pneumococcus serum protective experiments with white mice offers the best method. According to Cole, 0.2 c.c. of serum should protect against 0.1 c.c. (Type I) or 0.01 c.c. (Type II) of a broth culture (of which 0.000001 c.c. kills mice) when culture and serum are injected simultaneously. Protection experiments can also be applied to streptococcus serum.

On the whole the methods are far from satisfactory, titration of one antibody does not necessarily give us any information as to the content in other antibodies. Where many representative types are employed, the balance not only as regards one antibody, but possible variations in comparative content of different antibodies against the individual strains still further complicates matters as well as our insufficient knowledge of the actual immunological relationships of these strains. Protection tests are satisfactory where the bacterium

is truly septicemic for the test animal, less so where invasion is less marked (meningococci) and of least value where death is due essentially to endotoxins. Protection tests, furthermore, only give us information concerning the strain used, and in the case of meningococci, for instance, we know little as to cross-protection with strains having little or no agglutinative relationship. Furthermore, there is only partial knowledge as to the relation of antibody content to protection and finally to therapeutic effect in man.

**Bleeding.**—Bleeding for therapeutic sera are made with trocar and rubber tube into 2-liter Erlenmeyer flasks having a large flat wire egg-beater to support the clot. After the flask is about half-full, it is tilted on the side where the wire is inserted. The flasks may be stood up after the clot is firm. The serum separating after twenty-four and forty-eight hours is drawn off with siphon. All operations must be aseptic.

**Serum.**—The collected serum is placed in a sterile vessel and trikresol added drop by drop, with vigorous stirring to prevent precipitation, until 0.4 per cent. is added. In two to three weeks, after which further fibrin separation usually ceases, it is passed through a Berkefeld filter to remove any contamination occurring at the time the trikresol was added. The trikresol will prevent any multiplication of the contaminants prior to filtration. Instead of trikresol one may add several cubic centimeters of chloroform per liter and shake, the excess settles out with any separated fibrin and is avoided when the serum is drawn up in the bottling apparatus. The sera must be kept in the refrigerator at all times and tested to determine its sterility before being issued.

**Concentration.**—This is not practised as it is with the antitoxins. To test the different fractions and determine which to use is, to a great extent, impracticable till we have better methods for standardization and a clearer knowledge as to what antibodies are essentially of therapeutic value.

Gay and Chickering starting with the known fact that when antigen and immune sera combine to form a precipitate, other antibodies than the precipitins may be carried down, have devised a method for the concentration of the protective substances in pneumococcus serum. They find that the precipitate following the mixture of antipneumococcus serum and pneumococcus extract contains the agglutinins and protective substances, which can be extracted by treating the precipitate with dilute sodium carbonate at 42° C. This method is not practicable for general use.

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## CHAPTER XI.

### COMPLEMENT-FIXATION: THE TECHNIC OF THE TEST AND ITS PRACTICAL APPLICATIONS.

THE fact that the blood serum of individuals who have been inoculated with certain antigens (substances capable of producing antibodies) contains substances (antibodies of the amboceptor type) (see Chapter X) each of which after uniting in certain proportions with its specific antigen will combine with or fix a third substance found in all sera (complement), has been made use of in a practical way for determining the presence of either antigen or antibody in suspected infections. Neither antibody alone nor antigen alone can fix the complement, but both substances must be present in order to obtain fixation of complement. The result of such a reaction is usually invisible.

When red blood cells are used as an antigen, the resulting antibody in combination with its antigen, the red blood cells, fixes complement with the consequent breaking up of the altered red blood cell (lysis) used and the passing of its hemoglobin into the outer medium (serum or physiological salt solution). This causes the originally opaque fluid to become a transparent red. This phenomenon is called hemolysis and it is made use of as an indicator in the complement-fixation tests, when it is known as the hemolytic system.

Thus, when a bacterial antigen is mixed with serum containing the specific antibody for that antigen, and fresh serum containing complement is added all in definite and suitable proportions and the mixture is allowed to stand for a certain time at a certain temperature, there is a union of the antigen and antibody and all of the complement is absorbed. Such an antigen gives no visible sign of its altered condition but we may show that the complement has been absorbed by adding to the mixture an emulsion of red blood cells together with inactivated (heated at about 56° C. for half an hour to destroy the complement) hemolytic serum, when no hemolysis will take place because there is no free complement.

The lytic power of any serum therefore depends on the presence in the serum of two components, one of which is thermostable and specific, the other thermolabile and non-specific. The thermostable component is, according to Ehrlich's side-chain theory, an antibody of the third order, the amboceptor type, and is known as immune body, amboceptor, substance sensibilatrice (Bordet) or sensitizer. The thermolabile activating component of serum is a normal constituent of all fresh serum and is known as complement, alexin (Buchner and Bordet), or cytase (Metchnikoff).

The *hemolytic system* consists of three substances: erythrocyte suspension, homologous immune serum, and complement; and the presence

of all three in definite proportions is necessary for complete hemolysis. Erythrocytes to which sufficient hemolysin has been added to prepare them for complete dissolution by complement are called sensitized erythrocytes.

**DIAGRAMMATIC REPRESENTATION OF REACTION.**—*Explanation of Diagram.*  
—If complement is bound by the interaction of antigen and amboceptor it cannot join sensitized erythrocytes to form the hemolytic system; hence the latter is incomplete and hemolysis does not occur (Fig. 71, Positive Complement-fixation Test). If antigen is mixed with an immune serum that is heterologous instead of homologous, *i. e.*, the serum from an animal immunized with an antigen unrelated to the first antigen, then complement instead of being bound is still free and sensitized erythrocytes are hemolyzed (Fig. 71, Negative Complement-fixation Test).

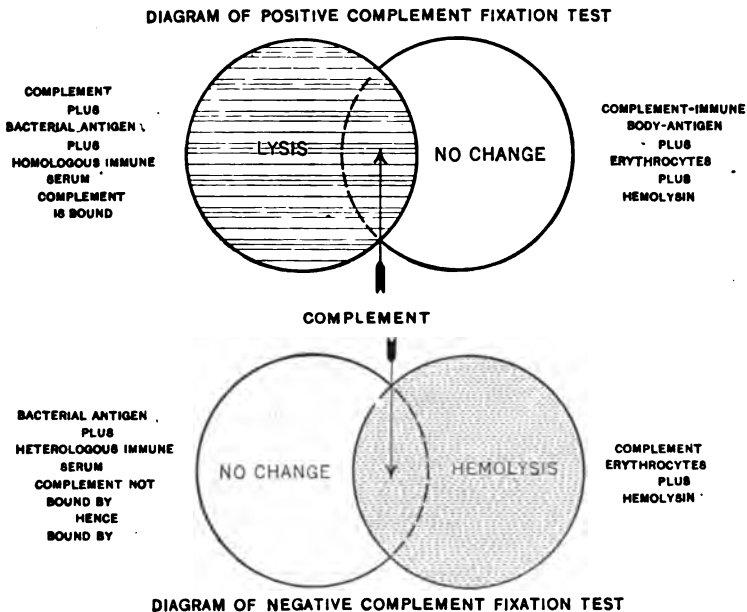


FIG. 71

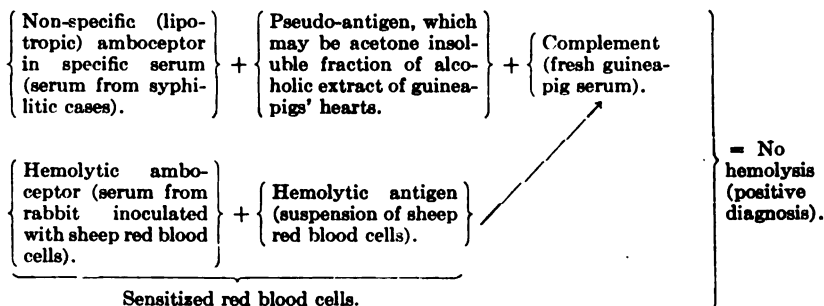
The chief reactions used in this test, as well as the whole test, may also be expressed by equations as follows:

**COMPLEMENT-FIXATION EQUATIONS.**

- I. Susceptible animal + specific antigen = Specific (microbial, hemolytic, etc.)  
amboceptor serum (Homologous serum)
- II. Microbial amboceptor +  $\left\{ \begin{array}{c} \text{microbial} \\ \text{or} \\ \text{pseudo-} \end{array} \right\}$  antigen + complement (fresh guinea-pig serum) =  $\left\{ \begin{array}{l} \text{Fixation} \\ \text{of comple-} \\ \text{ment with} \\ \text{no visible} \\ \text{reaction.} \end{array} \right.$
- III. Hemolytic amboceptor + hemolytic antigen + complement =  $\left\{ \begin{array}{l} \text{Fixation of comple-} \\ \text{ment indicated} \\ \text{by hemolysis.} \end{array} \right.$   

Hemolytic system or indicator.

## TEST AS EMPLOYED IN THE DIAGNOSIS OF SYPHILIS.



*Historical Note.*—The complement-fixation test has been developed from the following classical experiment of Bordet and Gengou (1901):

Typhoid bacteria (antigen), inactivated typhoid-immune serum (amboceptor), and normal serum (complement) were mixed in a test-tube; after an interval washed erythrocytes and inactivated homologous immune serum (hemolysin) were added. No hemolysis occurred, indicating that the complement had been fixed by the interaction of antigen and amboceptor, whereby the hemolytic system was left incomplete.

Wassermann and others have applied this method in measuring the amboceptor content of specific sera, the first practical application of its use being in the diagnosis of syphilis. Here it is known as the Wassermann reaction. As carried out in the diagnosis of syphilis the reaction was found to be due not to a specific antibody but to a lipid.

The complement-fixation may be applied to the identification of an unknown organism or other protein material that is tested as antigen against known immune serum, or it may be applied to the identification of unknown amboceptor in an immune serum that is tested against known antigen, inhibition of hemolysis showing that the antigen and immune serum are homologous. Complement-fixation is used therefore (1) in the diagnosis of various infectious diseases (for example, syphilis, gonococcus infections, and glanders), (2) in the differentiation of proteins, (3) in the standardization of some immune sera (for example, antigonococcus and antistreptococcus horse sera), and (4) in helping to establish the etiology of infectious diseases.

**Technic.—Preparation of Reagents.**—The *reaction of glassware and reagents* has been found to have an important bearing on the accuracy of the test, acidity or alkalinity giving rise to false reactions, positive or negative according to the degree of acidity or alkalinity. All new glassware should be neutralized by standing overnight in 1 per cent. hydrochloric acid. Then, like previously neutralized glassware, it is washed in tap-water, thoroughly rinsed in hot distilled water, and sterilized. The distilled water and salt solution used in the preparation of reagents and in the performance of the test are tested for neutrality to phenolphthalein. *Physiological salt solution*, 0.85 per cent. to 0.9 per cent., is used in blood-washing, in diluting other reagents, and in the performance of the test.

**Immune Serum.**—The method of withdrawing *immune serum* from an animal depends on the animal and the purpose of bleeding. In

the case of a horse, the bleeding is made from the jugular vein. Rabbits are bled from the marginal ear-vein if only a few cubic centimeters (less than 5 c.c.) of serum are required. If a large amount is required, the rabbit is etherized, tied on a board made for that purpose, and under aseptic precautions bled to death from the carotid artery. A cannula may be inserted in the artery and the blood allowed to flow into one or more tubes. To obtain the maximum amount of serum, the tube of blood should be slanted at room temperature until coagulation has taken place. In obtaining blood for complement-fixation tests on human beings venous *puncture* is the most satisfactory method. The drawing of sufficient blood from finger or ear-lobe is a tedious process and the blood cells are apt to be somewhat broken, so that the serum is tinged with hemoglobin and rendered unfit for testing.

Blood is most easily obtained from the median basilic vein of the elbow. The arm should be rendered aseptic by the usual method of scrubbing with soap and water and the application of a 1 to 2000 bichloride of mercury pack for ten minutes. The site of puncture should then be rubbed with alcohol and ether. A ligature is placed above the elbow sufficiently tight to fill the vein, but not tight enough to impede the arterial circulation. A sterile needle is then introduced and 5 to 10 c.c. of blood allowed to flow into a sterile test-tube, which is corked and left slanted at room temperature until the blood is firmly clotted. The tube should then be placed in the ice-box until serum has separated. If it is necessary to mail a blood specimen, serum only should be sent, as lysis of the blood cells would be caused by the heat and shaking to which the specimen would be exposed in transit. The serum should be mailed in a sealed ampoule or tightly corked tube.

All immune serum is separated from the red blood cells and fibrin of the blood by centrifugalization before coagulation has taken place, or, better, by pipetting or pouring from the clot after coagulation. Serum thus removed may be entirely freed from erythrocytes by centrifugalization. Serum should be removed from the clot before spontaneous lysis of the blood cells occurs, as hemoglobin has the power of fixing complement in itself and a hemolyzed specimen of serum, one containing hemoglobin, is *anticomplementary*, *i. e.*, it *inhibits hemolysis* without the presence of a specific antigen, hence cannot be tested for a specific amboceptor. Serum, both before and after removal from the clot, should be kept in a cool place, at a temperature not higher than 0° C., as the antibody content weakens more rapidly at a high temperature than at a low. For practical purposes *serum is best preserved by freezing*.<sup>1</sup> The addition of a preservative is not advisable, as the accuracy of the test may thereby be invalidated. Contamination of an immune serum should be avoided, as it may result in an anticomplementary and non-specific action of the serum. *All immune serum before use in tests should be inactivated, i. e., heated for one-half hour at 56° C., to destroy complement and serum components that might give rise to a non-specific fixation.*

**Antigen.**—The method of preparing antigen depends on the nature of the test to be made. Each antigen is described in detail below. A

<sup>1</sup> Serum is perfectly preserved by evaporating to dryness in a vacuum desiccator, but the procedure is complicated.

bacterial antigen may be prepared as in the original method of Bordet-Gengou, by suspending in physiological salt solution a twenty-four-hour agar growth of the bacterium, making a rather concentrated emulsion. Bacterial extracts give more specific results than emulsions unless the emulsions be made from the organisms dried *in vacuo* after being treated with alcohol and ether. Olitsky and Bernstein, among others, have shown that if antigens are made from cultures grown on serum media non-specific complement-fixing bodies may be found in them. The best method of preparation depends on the organism.

**Complement.**—Not all fresh serum has the power of reactivating the serum of an alien species, but that of the guinea-pig has unusual power in this respect; hence guinea-pig serum is generally used for complement in hemolytic work. There is a wide variation in the activating power of guinea-pig serum, and also in its power of combining with antigen and immune serum in the complement-fixation reaction. It is advisable to use the pooled serum of at least three pigs, in order to obtain complement of average activating and combining properties.

Guinea-pigs that have been used for antitoxin tests and other purposes may be of use in complement-fixation work after a rest of three months, but their serum is less apt than that of unused pigs to be of normal activating power. In the complement-fixation test for glanders pigs that have been inoculated with horse serum must never be used, as substances are formed that cause a reaction with the horse serum that is being tested for glanders and the test is unsatisfactory; serum controls in themselves inhibit hemolysis and no reading can be made of a specific reaction. Gravid pigs should not be used for complement, as their serum is apt to be weak in activating power.

To obtain complement, guinea-pigs may be bled from the heart by aspiration and used again after several weeks' rest, or they may be bled to death. In this laboratory the pigs, after being stunned by a blow at the base of the skull, are bled from the throat<sup>1</sup> into Petri dishes, which are left at room temperature until the serum begins to separate and then set in the ice-box for several hours. The serum is drawn off with a capillary pipette and centrifuged if not free from blood cells. Before being pooled, the serum from each pig should be tested separately for natural hemolysin, which is occasionally present, and for activating power. Serum containing natural hemolysin or serum of weak activating power should be discarded. Complement deteriorates rapidly if exposed to sunlight or to a warm temperature (over 70° F.); if kept in the ice-box (at a temperature below 17° C.) it is good for at least twenty-four hours. Complement may be preserved for several weeks by freezing. Its activating power is retained, but its capacity for being bound may weaken, so that it is not considered reliable. In our experience complement frozen for one week is as good as perfectly fresh complement.

*Complement is used in a 10 per cent. dilution made with physiological salt solution (see titration tables below).*

<sup>1</sup> Care must be taken not to cut the esophagus, as the stomach contents might render the serum unfit for use in complement-fixation work.

**Erythrocytes** from sheep, goat, man, ox, or other animal may be used, and they must be washed free from serum, with physiological salt solution. In our laboratory sheep's cells, washed six times, are used, in a 5 per cent. suspension. A sheep is bled from the jugular vein into a sterile bottle or flask containing glass beads, and before the blood coagulates it is thoroughly shaken for the purpose of defibrination.

Blood is washed in the following manner:

In washing a small amount of blood it is convenient to put 2 to 4 c.c. in a 15-c.c. graduated centrifuge tube. The tube is filled with physiological salt solution and a mixture of blood and saline is effected by means of a pipette. The volume of saline should be three times that of the blood, otherwise the washing is not thorough. The blood is rapidly centrifugalized long enough for the erythrocytes to fall to the bottom of the tube. The supernatant fluid is decanted or drawn off with a pipette attached to a pressure pump or fitted with a rubber bulb. Physiological salt solution is again mixed with the cells and the tube centrifugalized. This process is repeated until the blood cells are entirely free from serum. In most laboratories three washings are considered sufficient, but we have found that after even four washings the serum is not always completely removed. Hence we make it a *rule* to *wash six times*. After the last washing the level of the erythrocytes is marked on the tube, before the cells have been disturbed by the removal of the supernatant fluid. The amount of blood per centrifuge tube, the speed and duration of centrifugalization should always be the same, at least for the last washing, in order that the packing of the cells be uniform day by day.

Blood that has been drawn for over two days in summer or three days in winter is unsuitable for hemolytic work, as the resistance of the erythrocytes weakens on standing and hemolysis occurs rapidly, so that perfect balance of the hemolytic system cannot be obtained. In places where it is inconvenient to obtain sheep's cells, human erythrocytes are frequently used. An advantage in the human system arises from the fact that by its use one error in the test is avoided. Human serum frequently contains natural antisheep amboceptor and the excess of hemolysin introduced into the hemolytic system when such a serum is being tested may, according to some investigators, cause a weakening or loss of a positive reaction. In our experience this error rarely or never occurs in the Wassermann reaction, owing to our practice of reading the reaction as soon as controls are completely hemolyzed; hence the use of a hemolytic system other than sheep or the absorption of the natural hemolysin from the serum is not necessary in Wassermann work. In tests with bacterial antigens where the fixation of complement is less firm than in the Wassermann reaction the native antisheep amboceptor in human serum may be a source of error.

**Hemolysin** is obtained by successive inoculations of a rabbit (or other experimental animal) with the type of red blood cell to be used in tests. Inoculations may be made intraperitoneally or intravenously. Owing to the frequency of abscesses after intraperitoneal inoculations the intravenous method is preferable. In this laboratory, rabbits are inoculated intravenously at intervals of two days with fresh, thoroughly washed sheep's cells in a 50 per cent. suspension in physiological salt solution, the doses being 2 c.c., 4 c.c., and 6 c.c. On the ninth day after

the last inoculation the rabbit is bled to death from the carotid. The serum is put up in small bottles or ampoules and heated for one-half hour at 56° C. on three successive days, to destroy the complement and to insure sterilization. Hemolytic sera of high titre (1 to 3000) may be produced by this method.

**Standardization of Reagents.**—An accurate daily standardization of the hemolytic system is very important for reliable results, and this may be accomplished by means of a hemolysin or a complement titration. In the former, varying amounts of hemolysin are incubated with a constant amount of complement and erythrocyte suspension. In a complement titration the amounts of complement are varied and hemolysin is constant. In most laboratories it is considered *preferable to follow a hemolysin titration*. Preliminary titrations of new hemolytic immune serum are made in dilutions of 1 to 100, 1 to 1000, etc., to determine the dilution of hemolysin in which 0.05 c.c. is one unit. *By a unit of hemolysin is meant the smallest amount that gives complete hemolysis of 0.1 c.c. of a 5 per cent. sheep's cell suspension in the presence of an excess of complement after one hour's incubation in a 37° C. water-bath.*

Once standardized the hemolysin is used in the same dilution every day. The technic of the titration is given below. The volume, like that of all our complement-fixation tests, is one-tenth the volume of the classical Wassermann—*i. e.*, 0.5 c.c., instead of 5 c.c. After thorough shaking the titration is incubated one hour in the water-bath at 37° C., and examined frequently to determine the rapidity of the reaction, according to which from two to three units are used in the test. As hemolysin is stable and fresh, and erythrocytes from a healthy sheep vary little in resistance, such a titration indicates sufficiently for practical work the value of the complement. If the complement is normally active and free from hemolysin, the erythrocytes fresh and thoroughly washed, the saline solution isotonic and all reagents accurately diluted, hemolysis at the end of the hour's incubation is complete (Plate V, *e*) in the first six tubes, nearly complete (Plate V, *d*) in the seventh, partial (Plate V, *c*) in the eighth, slight in the ninth, and lacking (Plate V, *a* and *b*) in the last four tubes. Then since 0.05 c.c. is the unit of hemolysis, 0.1 c.c. is, in general, used to sensitize 0.1 c.c. of erythrocytes. The interpretation of a hemolysin titration can be learned by experience only.

#### HEMOLYSIN TITRATION.

Number of tube.	Hemolysin in standard dilution, c.c.	10 per cent. complement. c.c.	5 per cent. erythrocyte suspension. c.c.	0.85 per cent. saline. c.c.
1	0.1	0.1	0.1	0.0
2	0.09	0.1	0.1	0.21
3	0.08	0.1	0.1	0.22
4	0.07	0.1	0.1	0.23
5	0.06	0.1	0.1	0.24
6	0.05	0.1	0.1	0.25
7	0.04	0.1	0.1	0.26
8	0.03	0.1	0.1	0.27
9	0.02	0.1	0.1	0.28
10	0.01	0.1	0.1	0.29
11	0.1	0.0	0.1	0.3
12	0.0	0.1	0.1	0.3
13	0.0	0.0	0.1	0.4

Incubated for one hour in the water-bath at 37° C.

## PLATE V



- a.* Complete absence of hemolysis before settling of cells.
- b.* Complete absence of hemolysis after settling of cells.
- c.* Partial hemolysis.
- d.* Nearly complete hemolysis.
- e.* Complete hemolysis.





## COMPLEMENT TITRATION.

Number of tube.	10 per cent. complement. c.c.	Hemolysin in standard dilution. c.c.	5 per cent. erythrocyte suspension. c.c.	0.85 per cent. saline. c.c.	Incubated for one hour in the water-bath at 37° C.
1	0.1	0.05	0.1	0.25	
2	0.09	0.05	0.1	0.26	
3	0.08	0.05	0.1	0.27	
4	0.07	0.05	0.1	0.28	
5	0.06	0.05	0.1	0.29	
6	0.05	0.05	0.1	0.3	
7	0.04	0.05	0.1	0.31	
8	0.03	0.05	0.1	0.32	
9	0.02	0.05	0.1	0.33	
10	0.01	0.05	0.1	0.34	
11	0.1	0.0	0.1	0.3	
12	0.0	0.05	0.1	0.35	
13	0.0	0.0	0.1	0.4	

## ANTIGEN TITRATION.

Number of tube.	Immune serum c.c.	Antigen. c.c.	10 per cent. complement. c.c.	0.85 per cent. saline. c.c.	Incubated one-half hour in the water-bath at 37° C.	Sensitized erythrocyte suspension. c.c.	Incubated one hour in the water-bath at 37° C.
1	0.01	0.25	0.1	0.0		0.2	
2	0.01	0.2	0.1	0.0		0.2	
3	0.01	0.15	0.1	0.05		0.2	
4	0.01	0.1	0.1	0.1		0.2	
5	0.01	0.05	0.1	0.15		0.2	
6	0.01	0.025	0.1	0.2		0.2	
7	0.0	0.4	0.1	0.0		0.2	
8	0.0	0.3	0.1	0.0		0.2	
9	0.0	0.2	0.1	0.05		0.2	
10	0.0	0.1	0.1	0.1		0.2	
11	0.0	0.05	0.1	0.15		0.2	
12	0.02	0.0	0.1	0.2		0.2	

If an exact determination of *complement* value is desired the hemolysin titration may be followed by a *complement titration* as given above. *The unit of complement is the smallest amount that completely hemolyzes 0.1 c.c. of 5 per cent. erythrocyte suspension sensitized by one unit of hemolysin.* If the balance of the hemolytic system is to be obtained by varying complement instead of hemolysin, two units of hemolysin instead of one unit should be used in titrating complement. In our opinion more uniform and accurate results are secured by varying hemolysin than by varying complement.

*Antigen.*—There are two requirements for a good antigen, a *long range* and *specificity*.<sup>1</sup> The range of an antigen is determined by mixing varying amounts of the antigen with a constant amount of a previously tested homologous immune serum, a constant amount of complement and a constant amount of sensitized erythrocyte suspension. The specificity of an antigen is determined by using heterologous instead of homologous immune serum in the titration. The technic employed in this laboratory in the titration of an antigen is given above. Readings are made after the erythrocytes have settled, and meantime the titration should be kept in the ice-box to prevent a continuation of hemolysis. An immediate reading may be made by centrifugalizing the tubes. If

<sup>1</sup> The range of an antigen is the difference between the anticomplementary dose (the smallest amount of antigen that is in itself inhibitory) and the minimum fixing dose, the antigen unit.

fixation is complete through 0.025 c.c. (tube 6) a 10 per cent. solution of the antigen should be titrated in the same manner to determine *the unit of antigen, which is the smallest amount that, with two units of homologous immune serum* (or 0.01 c.c. of a human serum which has given a + + + + reaction), *gives complete fixation of complement*. The anti-complementary dose is the smallest amount of antigen that is in itself inhibitory. The longer the range of the antigen the greater is the probability of success in diagnosis. An amount of antigen that fixes complement completely with a serum of high antibody content may give incomplete or no fixation with a serum of low antibody content. Hence in making diagnostic tests, where the detection of even a small amount of antibody is desired, it is advisable to use much more than one unit of antigen. *As the maximum amount of antigen that may be used with safety is one-fourth the anticomplementary dose an antigen of long range is necessary*. If in the table just given inhibition is complete (Plate V, *a* and *b*) in the first five or six tubes, hemolysis is complete (Plate V, *e*) in tubes 8 to 12, and there is very slight inhibition (Plate V, *d*) in tube 7 only, then 0.1 c.c. is the amount of antigen to be used in diagnostic tests. *Antigen should always be so diluted that 0.1 c.c. may be used*. If one-fourth the anticomplementary dose gives complete fixation with a heterologous immune serum the antigen is non-specific and unsuitable for tests. *Occasionally an antigen is lytic for erythrocytes*. In such a case tubes containing the largest amount of antigen show more hemolysis than those containing less. The fixation curve instead of dropping (Fig. 72, *a*) first rises and then drops again (Fig. 72, *b*). *If a lytic antigen is also anticomplementary and has a long fixation range it may be used, otherwise it should be discarded*.

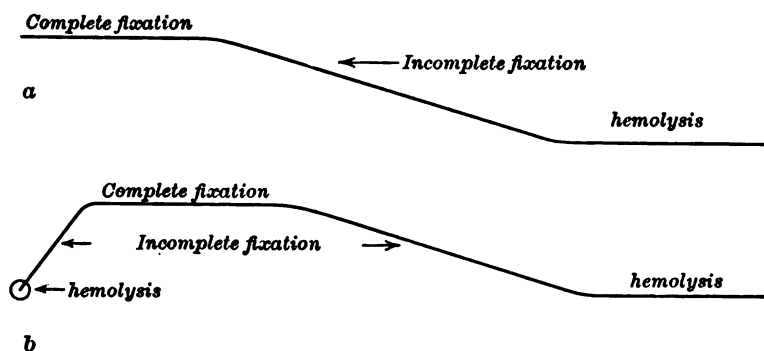


FIG. 72

Number of tube.	Patient's serum. c.c.	Antigen in standard dilution. c.c.	TEST.		Sensitized erythrocyte suspension. c.c.	Period for fixation.	Incubated until hemolysis is complete in tubes 3 to 6.
			10 per cent. complement.	0.85 per cent. saline.			
			c.c.	c.c.			
1	0.02	0.1	0.1	0.1	0.2		
2	0.01	0.1	0.1	0.1	0.2		
3	0.04	0.0	0.1	0.2	0.2		
4	0.02	0.0	0.1	0.2	0.2		
5	0.0	0.2	0.1	0.0	0.2		
6	0.0	0.1	0.1	0.1	0.2		

A *test for diagnosis* is set up as given above. Fixation of complement may be allowed to take place for one-half hour in a water-bath at 37° C., for one hour in the incubator at 37° C., at room temperature for four hours or more<sup>1</sup> or in the ice-box for from four to eighteen hours. Whichever method is employed an antigen titration fixed by the same method should be followed, as the antigenic range varies with the time and temperature allowed for fixation. Positive and negative control sera should always be included in the test. Citron's standard for the strength of a complement-fixation reaction is used by this laboratory in the reading of all tests (Plate V):

Complete absence of hemolysis in tubes 1 and 2 = + + + +, very strong positive.

Complete absence of hemolysis in tube 1, faint hemolysis in tube 2 = + + +, strong positive.

Complete absence of hemolysis in tube 1, complete or nearly complete hemolysis in tube 2 = + +, positive.

Partial hemolysis in tube 1, complete or nearly complete hemolysis in tube 2 = +, weak positive or doubtful.

Nearly complete hemolysis in tube 1, complete hemolysis in tube 2 = =, doubtful.

Complete hemolysis in all tubes = -, negative.

The distinction between + + and + is very important, as the latter reaction, we have heard, is frequently non-specific. We therefore make a positive diagnosis on only + +, + + +, or + + + + reactions.<sup>2</sup>

An *antibody content titration* is made for the purpose of measuring more accurately than in the diagnostic test just described the amboceptor content of an immune serum. Our technic is given in table below. The serum is first titrated in a 10 per cent. solution; if fixation is complete through 0.01 c.c. a similar titration of a 1 per cent. dilution is made. The amount of antigen used is double the antigen unit; for example, if 0.05 c.c. of a 10 per cent. solution of antigen is the smallest amount giving complete fixation with a homologous immune serum, 0.1 c.c. of that dilution is used in the antibody content titration. The reading is made as in an antigen titration, after the erythrocytes have settled. The antigen and serum controls (tubes 11 and 12) should, of course, be hemolyzed. An *antibody unit* is the smallest amount of serum that with two units of an homologous antigen gives complete fixation of complement. The number of antibody units per cubic centimeter may be calculated by dividing 1 c.c. by the minimum fixing dose; that is, if 0.05 c.c. of a 1 in 10 dilution is the antibody unit, the number of antibody units per cubic centimeter equals  $\frac{1}{0.05} = 200$ . An antibody content titration is used for the standardization of antimicrobial sera, such as the antistreptococcus and antistaphylococcus horse sera. The power of a serum to fix complement

<sup>1</sup> In our experience, room temperature is not desirable for fixation, as complement may deteriorate and the hemolysis of control tubes cannot be relied upon even though the hemolytic system when standardized is active.

<sup>2</sup> A + Wassermann reaction following the treatment of a case that has given a + +, + + +, or + + + + reaction is considered *positive* and indicates the need of further treatment.

is not, however, always parallel with its protective or therapeutic power. Besredka found that antistreptococcus serum of high protective power had little complement-fixing power, and *vice versa*.

#### ANTIBODY CONTENT TITRATION.

Number of tube.	*Immune serum. Diluted 1 to 10 (etc.). c.c.	Antigen in dilution determined by titration. c.c.	10 per cent. complement. c.c.	0.85 per cent. saline. c.c.	Sensitised erythrocyte suspension. c.c.
1	0.1	0.1	0.1	0.0	0.2
2	0.09	0.1	0.1	0.01	0.2
3	0.08	0.1	0.1	0.02	0.2
4	0.07	0.1	0.1	0.03	0.2
5	0.06	0.1	0.1	0.04	0.2
6	0.05	0.1	0.1	0.05	0.2
7	0.04	0.1	0.1	0.06	0.2
8	0.03	0.1	0.1	0.07	0.2
9	0.02	0.1	0.1	0.08	0.2
10	0.01	0.1	0.1	0.09	0.2
11	0.0	0.2	0.1	0.0	0.2
12	0.2	0.0	0.1	0.0	0.2

Incubated one-half hour in the water-bath at 37° C.

Incubated one hour in the water-bath at 37° C.

**The Wassermann Reaction.**—The antigen originally employed by Wassermann, Neisser, and Bruck, and still preferred by some workers, consists of a saline extract of liver from a syphilitic fetus. The finely divided tissues are mixed in the proportion of one to four with normal salt solution, to which 0.5 per cent. carbolic has been added, agitated at room temperature for twenty-four hours, centrifuged, and the supernatant fluid drawn off into sterile vessels and kept in the ice-box until needed. Since the cultivation of the *Treponema pallidum* has been made possible, antigens have been made from the organisms in pure culture instead of from luetic tissue; but the results have, on the whole, been less satisfactory, *i. e.*, fewer cases of syphilis give a positive Wassermann reaction with a *Treponema pallidum* antigen than with a luetic tissue antigen, or even with a lipid antigen from normal tissues. The exact nature of the antigen that produces the antibodies (called by Citron *Luesreagin*) taking part in the Wassermann reaction is unknown. It appears to be neither the pure spirochetes, nor a pure lipid substance. Although a pure lipid cannot stimulate the production of antibodies when inoculated into an experimental animal, it reacts *in vitro* with the *Luesreagin* in the blood of the syphilitic. The Wassermann reaction then is a lipotropic or lipoidophilic reaction and not due to the interaction of specific antigen and antibody. An extract of heart, liver, or kidney in 96 per cent. alcohol may be used. Some serologists recommend the addition of cholesterin to a crude alcoholic extract; such an antigen gives a higher percentage of positive reactions than most other antigens, but its reliability has not yet been established.

In our experience a cholesterinized antigen is of special value in determining the effectiveness of antiluetic treatment, as old cases of syphilis that are or have recently been under treatment are more apt to react with this antigen than with a crude alcoholic antigen. In making a fresh diagnosis it is not wise to depend on a cholesterinized antigen alone, since cases of scarlet fever, leprosy, and other non-syphilitic conditions have been found to react strongly with this antigen. These false positive reactions do not occur with a crude alcoholic antigen. A positive reaction is, however, presumptive of syphilis and a negative

reaction has more value in excluding a diagnosis of syphilis than has a negative reaction with the crude alcoholic antigen.

A safe and stable antigen is Noguchi's acetone insoluble fraction of beef heart, liver, or kidney, prepared by extracting macerated tissue with ten times the amount of absolute alcohol at room temperature for several days, filtering, evaporating the filtrate to dryness, taking up the residue with ether, treating the ethereal solution with five times its volume of acetone, and making a saturated solution of the precipitate in absolute methyl alcohol.

In our laboratory the most satisfactory results have been obtained with a crude alcoholic extract of guinea-pig hearts prepared as follows: hearts (from pigs bled to death for complement) are minced and washed in tap-water until free from blood and macerated in C. P. 96 per cent. alcohol in the proportion of 1 gram of heart to 5 c.c. of alcohol. Extraction is allowed to take place in the ice-box for at least three months and then in the incubator for a week. The extract is filtered through paper and kept in the ice-box, where it remains stable for at least a year. The titre of different antigens varies from 1 to 20 to 1 to 100. In diluting the antigen the first 10 c.c. of saline are added drop by drop and the remainder slowly, the mixture being effected by gently rotating the receptacle, not by shaking.

The table on page 198 gives the classical Wassermann technic. The readings are made according to Citron's standard. Our technic differs in three respects from Wassermann's: in the size of the test, in the amount of antigen used, and in the method of fixation. In our experience the results obtained through using all the reagents in one-tenth the amounts used by Wassermann are absolutely reliable, provided the test is accurately performed, and the saving in material is considerable. Another advantage in the small size is that a very small amount (about 0.2 c.c.) of the patient's serum is sufficient for a test. Concerning the amount of antigen to be used in the test, we have found the use of a constant amount to give the same results as the use of a varying amount. For fixation of complement a four-hour period in the ice-box is allowed instead of incubation, as it has been found that about 10 per cent. more positive reactions in cases of syphilis are obtained by this method, and without any apparent danger of obtaining false positive reactions. Though biologically non-specific the Wassermann reaction is clinically specific except, perhaps, in cases of leprosy, yaws, sleeping sickness, and scarlet fever.<sup>1</sup> With these exceptions a positive reaction indicates the presence of luetic infection, either active or latent. A positive reaction may be given in any stage of syphilis but is most apt to occur in the secondary stage. A negative reaction at any stage of the disease does not exclude the possibility of syphilis. Antiluetic treatment, especially mercury, frequently results in the reaction becoming negative, though the disease may still be active. Treatment with salvarsan and even with mercury and the iodides, may at first or after a few treatments cause a negative Wassermann to become positive for a time. A cure can only be pronounced when the reaction has remained negative for at least a year after intensive treatment and when it then still remains negative after a provocative treatment with salvarsan.

The ingestion of alcohol or the administration of an anesthetic within twenty-four hours of collection of the blood specimen interferes with

<sup>1</sup> With the use of the crude alcoholic antigens, in one-quarter the anticomplementary dose, we have never obtained false reactions in such cases ever by ice-box fixation.

the accuracy of the test, alcohol weakening the reaction and an anesthetic giving rise to false positive reactions.

Spinal fluids may give a positive reaction when the blood is negative in cases in which the brain or cord is involved, hence this fluid should always be tested when a negative report is received from a blood examination in which disease in such locations is suspected.

#### CLASSICAL WASSERMANN TEST.

Number of tube.	Lytic liver extract diluted. c.c.	Patient's serum. c.c.	10 per cent. complement. c.c.	0.85 per cent. saline. c.c.		Sensitized erythrocyte suspension. c.c.	
1	1.0	0.2	1.0	0.8		2.0	Incubated until
2	0.5	0.1	1.0	1.4	Incubated	2.0	control tubes
3	2.0	0.2	1.0	1.8	one hour.	2.0	are completely
4	2.0	0.0	1.0	0.0		2.0	hemolyzed.

To avoid error in the interpretation of the complement-fixation reaction, each diagnostic test should be accompanied by the following controls:

Serum controls	{	1. Duplicate diagnostic test to detect error in technic.
		2. Test for natural antishoop amboceptor to avoid false negative reactions.
		3. Test for anticomplementary unit.
		4. Test for lytic action upon cells alone.
Antigen controls	{	1. For specificity of antigen. { Diagnostic test with known positive human serum. Diagnostic test with known negative human serum.
		2. Test for anticomplementary unit.
		3. Test for fixation unit.
		4. Test for lytic action with complement when no amboceptor is used.

**The Complement-fixation Test for Gonococcus Infection.**—This method was first applied to the study of gonococcus infection by Müller and Oppenheim (1906). The technic developed by Schwartz and McNeil has been widely followed and the value of the test in the diagnosis of secondary gonococcus infections, especially in conditions in which a bacteriological diagnosis is difficult, as in arthritis, has been thoroughly established. A *polyvalent antigen*, one made from as many different strains of the gonococcus as possible, is essential.<sup>1</sup> A twenty-four-hour growth on salt-free veal<sup>2</sup> agar neutral to phenolphthalein is washed off with sterile neutral distilled water, autolyzed in a 56° C. water-bath for one hour, and in an 80° C. water-bath for one hour (to destroy any ferment that might render the antigen unstable), filtered through paper pulp and a Berkefeld filter,<sup>3</sup> and heated on three successive

<sup>1</sup> In this laboratory ten strains of gonococci isolated by Torrey are used in the preparation of the antigen.

<sup>2</sup> Medium from bob-veal is most desirable, made in the regular way but without salt.

<sup>3</sup> New Berkefeld filters are very alkaline, and before use for the filtration of bacterial antigens they should be boiled in distilled water at least three times for five minutes each time, and scrubbed thoroughly with a small brush in fresh water, after each boiling. After the filter is set up, hot, neutral, distilled water should stand in it for about five minutes. Then hot, neutral distilled water should run through under gentle pressure until the fluid is clear and neutral to phenolphthalein, when the filter is ready for use. Under high pressure a filter still alkaline might test neutral. After use, the filter should be boiled in distilled water, scrubbed, and dried in the air. A filter used for gonococcus antigen should never be used for any other bacterial antigen, unless it is first boiled in 1 per cent. sodium hydroxide solution and reneutralized.

days at 56° C. for one-half hour for sterilization. This antigen is stable for at least six months and is highly specific.<sup>1</sup>

Immediately before use the antigen must be made isotonic by the addition of one part of 9 per cent. saline solution to nine parts of the antigen. The test is performed in the usual way. The optimum period for fixation has been found to be six hours in the ice-box. Readings must be made with great care. A positive diagnosis should not be made unless 0.02 c.c. of serum gives complete inhibition of hemolysis, as serum containing heterologous amboceptors, for example, streptococcus, may give a + reaction with gonococcus antigen. Only gonococcus amboceptors give a ++, +++, or +++++ reaction. Cases of anterior gonorrheal urethritis and acute vulvovaginitis rarely give a positive complement-fixation test. A positive reaction is indicative of the presence or recent activity in the body of a focus of living gonococci. A positive reaction may persist for from six to eight weeks after a cure has been effected. Persistently negative results obtained through a considerable period of time indicate the probability of a cure.

**The Complement-fixation Test for Glanders.**—Complement-fixation has proved to be a valuable aid in the diagnosis of glanders. It is generally considered specific and more reliable than the agglutination test. In this laboratory the antigen is prepared from a twenty-four-hour growth of *B. mallei* on salt-free veal-agar 1.6 per cent. acid. The growth is washed off with sterile distilled water, autolyzed for six to eight hours at 80° C., filtered through paper pulp and a Berkefeld, and the filtrate sterilized at 56° C. on three successive days for a half-hour. This antigen, like the gonococcus and all other aqueous extracts, must be made isotonic before use. The test is performed like the others, but the optimum period for fixation has been found to be 6 to 18 hours in the ice-box. The New York Health Department condemns all horses that give a +++++ complement-fixation reaction when it is confirmed by the eye mallein test, while those that give a strongly ++ and +++ are suspected of having a slight glanders infection.<sup>2</sup>

**Streptococcus Infections.**—The value of complement-fixation in the diagnosis of streptococcus infections is still uncertain. In this laboratory fairly satisfactory results are obtained with a saline antigen prepared as follows: A twenty-four-hour growth on salt veal-agar neutral to phenolphthalein is washed off with 0.85 per cent. saline solution. The emulsion is heated at 60° C., one hour, left in the ice-box twenty-four hours, and centrifugalized. The supernatant fluid is used for tests, either water-bath or incubator being used for fixation. The test seems to be specific, but is far from perfected.

**Pertussis.**—Complement-fixation has been used for the determination of the etiological cause of whooping-cough and for the diagnosis of the disease. The results of many investigators, including ourselves,

<sup>1</sup> The specificity of a gonococcus antigen may be best determined by an antigen titration against antimeningococcus and antistreptococcus sera of high antibody content.

<sup>2</sup> At autopsy, macroscopic lesions are always shown by horses giving a +++++ complement-fixation test, rarely by those giving a ++ or a +++ reaction.



confirm the findings of Bordet, that the Bordet-Gengou bacillus is the etiological cause of whooping-cough. As to the diagnostic value of the test, reports vary. The figures given by the workers who use active serum are undoubtedly too high, as investigations in this laboratory have proved that active serum may give non-specific fixation with even a highly specific antigen. We have found the most satisfactory antigen, that is, the strongest and most specific, to be obtained by shaking and then autolyzing at 56° C. A twenty-four-hour culture on the Bordet-Gengou potato-blood-agar medium is scraped off with a platinum spud and put in sterile distilled water. The emulsion is shaken for about two hours, left in a thermostat at 56° C. for about eighteen hours, and centrifugalized. The supernatant fluid is used for tests, being made isotonic immediately before using. Water-bath or incubator is used for fixation; room temperature, at which some workers allow fixation to take place, gives such variable results as to be unsuitable for routine work. We obtain a positive reaction in about 50 per cent. of whooping-cough cases in the paroxysmal stage. The administration of whooping-cough vaccine may increase the strength of the complement-fixation reaction, but experiments with normal individuals have proved that vaccine in itself, in the absence of whooping-cough, does not bring about a positive reaction. Immune serum of high antibody content may be produced by the intraperitoneal inoculation of rabbits once a week with a live culture of the Bordet-Gengou bacillus, beginning with a dose depending on the condition of the rabbit. The height of immunity is usually reached through five or six inoculations; if other inoculations are given, the antibody content decreases. The rabbits are bled nine days after the last inoculation.

**Complement-fixation Test for Tuberculosis.**—The application of the Bordet-Gengou phenomenon to the study of clinical and experimental tuberculous infections in man and various animals has been repeatedly and variously tried. No method has as yet been devised which yields results in any way comparable in value to those afforded by the Wassermann reaction in syphilis. Antigens made from the various single and combined constituents of the tubercle bacillus, representing both the protein as well as the lipoid fractions, emulsions of living or dead tubercle bacilli and also the substances elaborated by the tubercle bacillus in many kinds of nutrient media have all been employed. When it is remembered that in tuberculous infections the usual antibodies are scant or wholly wanting in the serum it is not surprising that investigators have encountered such great difficulties in devising a satisfactory method. The very nature of the disease, involving as it does, even after arrest and healing, the presence of an infective focus, requires the greatest refinement of the technic in order that a discrimination can be made between latent or inactive infection and active infection or disease. The majority of the methods already recommended yield little or no information more than that elicited by proper tuberculin tests. The method of Besredka, in which the antigen is prepared from a culture on an egg medium has given good results in his hands and also with Bron-

fenbrenner. Miller and Zinsser, using the Wassermann technic with an antigen made by grinding living tubercle bacilli with dry salt and then emulsifying the ground bacilli, report encouraging results and go so far as to say that by this method they are able to detect active tuberculosis. Until further confirmation is forthcoming, the complement-fixation method in tuberculosis should be considered as being of scientific, rather than of clinical value. It is in too early a stage of development to be relied upon in either diagnosis or prognosis and it would seem that its greatest value might lie in the light it may shed upon the mechanism of tuberculosis immunity.

An excellent review of the literature is given by Stimson and by Miller.

**Meningitis.**—The complement-fixation method has been successfully applied in epidemic meningitis by Brück, but the diagnosis can more readily be made by the bacteriological examination of cerebrospinal fluid. Complement-fixation is a valuable means of differentiating strains of meningococci. The antigen used here for this purpose is prepared in the same manner as gonococcus antigen. Water-bath fixation is used for all titrations.

**Typhoid.**—Complement-fixation is a valuable method for corroborating the Widal. Its exact clinical value and specificity have not yet been established. The use of a highly polyvalent antigen is essential. Garbat's method of preparing the antigen is to wash off with sterile distilled water a twenty-four-hour growth on agar. Heat the emulsion at 60° to 70° C. for twenty-four hours, shake with glass beads for twenty-four hours, and centrifugalize until the supernatant fluid is absolutely clear. A positive reaction usually appears only after bacteria have disappeared from the circulation; it becomes stronger during convalescence and persists for several months afterward.

**Parasitic Skin Diseases.**—Kolmer and Strickler, corroborated by others, reported rather favorable results of fixation of complement in ringworm and favus.

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## CHAPTER XII.

### AGGLUTINATION AND PRECIPITATION OF MICRO-ORGANISMS AND THEIR PROTEINS.

#### THE NATURE OF THE SERUM SUBSTANCES CONCERNED.

By the phenomenon of agglutination is meant the aggregation into clumps of uniformly disposed microorganisms in a fluid because of the action of an homologous immune serum. If the organisms are motile they become immobile. Many other substances other than those in serum cause the agglutination of cells. We are only interested in these because they may cause confusion.

This phenomenon, while it had been noted by earlier observers (Charrin and Roger in 1889), was first extensively studied by Gruber and Durham in 1896, who determined that the serum of those passing through certain infections contained a specific substance (agglutinin) which caused the infecting organisms to clump. Several months later Widal reported that in typhoid fever the development of agglutinins could be used for diagnostic purposes. It was thus demonstrated by these studies and those of Grünbaum, Bordet and others that through agglutinins a new means was available for the identification of bacteria and in many cases the nature of the infecting organism causing disease.

As to the nature of these phenomena a number of theories have been advanced. There is a close analogy between agglutination and the flocculation of colloidal suspension by electrolytes. As in the case of the immune body, there is positive proof that the agglutinin combines directly with agglutinable substances in the bacterial body, the two bodies effecting a loose combination. But since a certain amount of sodium chloride or other inorganic salt is necessary (Bordet) it must be classed as a physicochemical reaction. Dead bacteria agglutinate as well but more slowly than living bacteria. The antigenic substance is designated as agglutigen and the antibody as agglutinin. Ehrlich considers that the agglutinin consists of a haptophore or combining atom group which is stable and of a ferment group which is labile (receptors of the second order, Fig. 68). The latter causes the phenomenon of agglutination.

In some types of infection there is a great accumulation of agglutinins in the blood. Thus in typhoid patients and convalescents distinct agglutination has been observed in dilutions of 1 to 5000, and this reaction persisted for months, though not, of course, in the same degree. Even normal blood serum, when undiluted, often produces agglutination through group agglutinins. But the specific agglutinins, which are formed only in consequence of an infection, are characterized by this,

that they produce agglutination even when the serum is highly diluted, and, furthermore, that after this dilution the action is generally specific—i. e., the high dilutions of cholera-immune serum agglutinate only cholera spirilla, of typhoid-immune serum only typhoid bacilli, etc. This specificity, however, for some bacteria such as the colon and dysentery group, is not always absolute, as the group agglutinins previously present may be in unusual amount.

It was formerly assumed that agglutination was a *prerequisite* for *bacteriolysis*. This, however, is not so, for both in cholera and in typhoid immunity bacteriolytic substances have been observed without agglutinins, and agglutinating substances without bacteriolysins.

**Characteristics of Agglutinins and Agglutinogen.**—The union of agglutinin with agglutinogen in bacteria is a physicochemical reaction, and is quantitative. By chemical means it is possible again to separate a portion of the agglutinin from bacteria saturated with it and use it to agglutinate bacteria anew. The amount of bacteria in the emulsion used to test the amount of agglutinin must therefore be known if a quantitative determination is desired. An emulsion one hundred times as dense as another would require one hundred times as much agglutinin to give an equally complete reaction.

Heating the serum above 60° C. injures the agglutinin but slightly, above 70° C. greatly, and above 75° C., destroys it.

Agglutinins changed by heat, acids, and other influences may become “agglutinoids,” which are comparable to toxoids, *e. g.*, agglutinating sera heated to a certain temperature lose their power to agglutinate but act upon bacteria so that they are unable to be agglutinated by active serum.

Heating the bacteria above 60° C. diminishes their agglutinability. Dreyer found that if a twenty-four-hour bouillon culture of *Bacillus coli* required 1 part of agglutinin to agglutinate it, then if heated to 60° C. it required 2.3 parts; if to 80° C., 18 parts; if to 100° C., 24.6 parts. He found the surprising fact that long heating of the culture restored to some extent its ability to be agglutinated by smaller amounts of agglutinins. Heated thirteen hours to 100° C., the culture was agglutinated by four parts.

Agglutinin does not dialyze through animal membranes. In dilute solution agglutinin slowly deteriorates. Dried, it lasts longer. It is precipitated with the globulins by ammonium sulphate. When a solution containing agglutinin is passed through a stone filter the first few cubic centimeters contain no agglutinin. The next contain a moderate amount and the remainder the same as the solution.

It is important to remember that in low dilutions of a serum agglutination may fail, while in higher dilutions agglutination may take place readily.

Weak and strong acids agglutinate bacteria, while medium acidity does not. Alkalis inhibit agglutination.

**The Development of Agglutinin.**—Experimental or natural infection of animals and men is followed in seven to ten days by an appreciable

development of agglutinin. This development is much greater in certain bacterial infections than in others.

**Group Agglutination.**—Many varieties of organisms have, among the different protein substances composing their bodies, some that are common to other microbes which are more or less allied to them (Fig. 73). If these substances are of the type that excite agglutinins, we have from an animal immunized by any one of them a serum acting on other organisms somewhat in proportion to the amount of agglutinin-producing protoplasm which they have in common with the infecting organism. These agglutinins, acting on substances common to other microorganisms which are generally but not always allied varieties are called, therefore, group agglutinins. Thus, in a case, the infecting paratyphoid bacilli type B were agglutinated 1 to 5700; typhoid bacilli, however, only 1 to 120, while paratyphoid bacilli type A were agglutinated only 1 to 10. In a case of typhoid fever an agglutination of paratyphoid type B occurred with a dilution 1 to 40, while typhoid bacilli were agglutinated with 1 to 300.

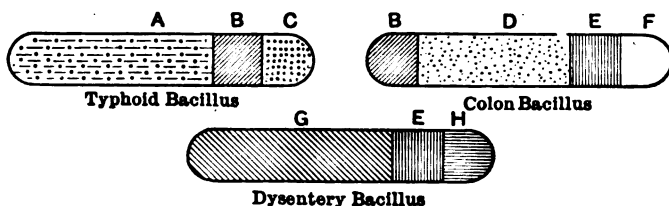


FIG. 73.—Specific and common agglutinins producing protoplasm.

The bacteria which are agglutinated by one and the same serum need not at all be related in their morphological or other biological characteristics, as at first assumed. Conversely, microorganisms which, because of the characteristics mentioned, are regarded as entirely identical are sometimes sharply differentiated by means of their agglutination. In other words, the "groups" arrived at by means of a common agglutination have no necessary relation to species as the term is usually employed, but only of chemical similarity. This is indicated by the diagrams in Fig. 73. The letters indicate chemical substances capable of stimulating the production of agglutinin and of combining with it when made. Thus both the typhoid and colon will stimulate B agglutinins and react to them, while type A agglutinins are produced only by the typhoid bacilli, and type D only by *B. coli*. Because of this lack of absolute specificity of the agglutination reaction the clinical *diagnosis of the type of infection or the absolute identification of bacteria* through the agglutination test can only be determined in those cases where the group agglutinins are not abundantly present. This suffices for some infections such as those caused by the typhoid bacillus and the cholera spirillum, but not for others such as those due to the colon group of bacilli. With the use of absorption methods given below the specific agglutinins can be separated in most cases from the group agglutinins.

**The Relative Development of Specific and Group Agglutinins.**—The study of a large number of series of agglutination tests obtained from young goats and rabbits injected chiefly with typhoid, dysentery, paratyphoid, paracol, colon, and hog-cholera cultures has shown that there is considerable uniformity in the development of the specific and group agglutinins. The specific agglutinins develop a larger amount of their total in the early days, being in the second week usually from five to one hundred times as abundant as the group agglutinins. Later the total amount of the group agglutinins tends to approach more nearly to that of the specific, and may reach as high as 20 to 50 per cent. In a number of tests carried out by us we found that many group agglutinins supplement specific ones in their action, causing by their addition an increased agglutinating strength. In our experience the variety of microorganism used for inoculation is, if equally sensitive, agglutinated in a higher dilution by the combined specific and group agglutinins produced through its stimulus, than any microorganisms affected merely by the group agglutinins. It is true that related bacteria were at times agglutinated in higher dilutions than the variety injected; this, if not due to greater sensitiveness, was caused by normal group agglutinins present in the animal before immunization. For this reason untreated horse serum is a very dangerous substance to use in differentiating the intestinal bacteria unless the serum is tested for group agglutinins. The great height to which the group agglutinins may rise is seen in the following table:

AGGLUTININ IN THE SERUM OF A HORSE INJECTED WITH PARADYSENTERY BACILLUS, CULTURE TYPE MANILA.

Culture.	After 18 injections.			After 21 injections.		
	1 : 3000	1 : 5000	1 : 10,000	1 : 3000	1 : 5000	1 : 10,000
Paratyphoid type Manila	++	—	—	++	++	++
Colon B. X.	++	++	—	++	++	++

The great amount of agglutinins acting upon the colon bacillus X. is remarkable. A serum is here seen to be acting in dilutions as high as 1 to 10,000 upon a culture possessing different characteristics from the one used in the injections.

Although a considerable proportion of the group agglutinins acting on colon bacillus X. was undoubtedly due to the stimulus of the injections of the paratyphoid culture, still a portion of them was probably due to the agglutinins developed by the stimulus of the absorbed intestinal bacteria. In the table given below is seen the marked accumulation of agglutinins which may occur in a normal horse before injections are begun:

Culture.	A young horse before inoculation.			
	1 : 100	1 : 500	1 : 1000	1 : 5000
Dysentery B., Japan	+	—	—	—
Paratyphoid, Mt. Desert	+	—	—	—
Paratyphoid, Manila	++	++	++	—
Colon B. X.	++	+	—	—

**The Relative Accumulation of the Group and Specific Agglutinins for the Organism Injected and for Allied Varieties.**—A test was carried out with different types of dysentery bacilli. For the Manila culture of Flexner, which is nearest to the colon in its characteristics, the specific agglutinins were, in the serum of an animal which had received injections of the Manila cultures, at the end of the fourth month five times as abundant as the group agglutinin acting on the Mt. Desert culture of Park, which represents a type lying between the Flexner and Shiga cultures. For the dysentery bacillus (Shiga) the development of agglutinins was the least (Fig. 74).

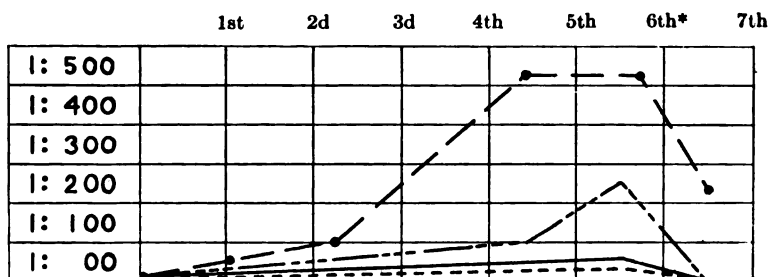


Fig. 74.—The rise and fall of common and specific agglutinins during seven months in a rabbit injected with the Manila culture.

- Colon bacillus X.
- Paradysentery type (Mt. Desert).
- ..... Paradysentery type (Manila).
- . . . . Dysentery type (Japan).

. Test dates for all four sera.

\* Injections stopped.

Another point of interest is that the proportional amounts of agglutinins from the different cultures varied at different times. If on tests made of a single bleeding we had attempted to draw conclusions as to the relative development of specific and group agglutinins between the cultures, we should have had an imperfect view. Many conflicting statements in literature are undoubtedly due to this lack of appreciation of the variability in the relative amount of these two types of agglutinins during a long process of immunization (Fig. 75).

**The Use of Absorption Methods for Differentiation between Specific and Group Agglutinins due to Mixed Infection and to a Single Infection.**

—It is now well established that if an infection is due to one micro-organism there will be specific agglutinins for that organism and group agglutinins for that and other more or less allied organisms. If infection is due to two or more varieties of bacteria, there will be specific agglutinins for each of the microorganisms and group agglutinins produced because of each of them.

The following experiments will illustrate these points:

A rabbit immunized to *B. typhi* agglutinated *B. typhi* 1 to 5000, *B. coli* 31 1 to 600. After saturation with *B. typhi* all agglutinins were removed for both microorganisms. A rabbit immunized to both *B. typhi* and *B. coli* 31 agglutinated *B. typhi* 1 to 4000, *B. coli* 31 1 to 1000. After saturation with *B.*

*typhi* the serum did not agglutinate *B. typhi*, but agglutinated *B. coli* 31 1 to 900. After saturation with *B. coli* 31 it failed to agglutinate *B. coli* 31, but still agglutinated *B. typhi* 1 to 3500. Some other strains of *B. coli* still agglutinated in 1 to 20 or more because many strains included in this group act as differently toward each other in respect to agglutinins as they do to the typhoid bacilli.

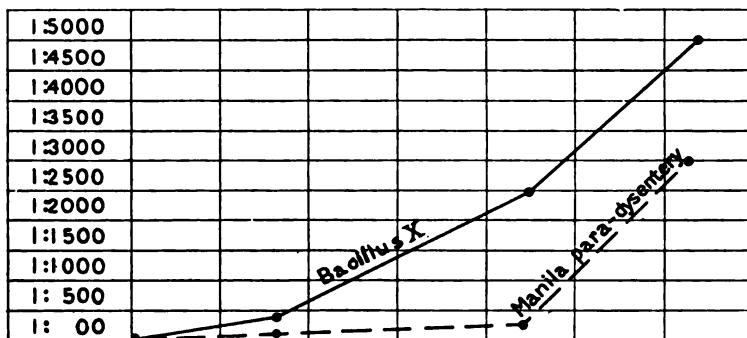


FIG. 75.—Similar conditions to those noted in previous chart, except that a young goat has been used for the injections of the colon bacillus X. The great accumulation of common agglutinins for the paradyseutery bacillus in the third month of the injections of the bacillus X is very striking.

. Tests made.

The following tables give the outcome of several experiments:

ABSORPTION BY THE TYPHOID BACILLUS OF GROUP AGGLUTININS ACTING UPON A NUMBER OF VARIETIES OF *B. COLI* WHICH WERE PRODUCED BY ANOTHER VARIETY OF *B. COLI*. AGGLUTINATION BY SERUM OF RABBIT IMMUNIZED TO *B. COLI* X.

	Before addition of typhoid bacilli.	After attempt at absorption with typhoid bacilli at 22° C.
Colon bacillus X . . . . .	6000	5000
Colon bacillus 1 . . . . .	500	20
Colon bacillus 2 . . . . .	500	30
Colon bacillus 3 . . . . .	250	30
Colon bacillus 4 . . . . .	250	10
Colon bacillus 5 . . . . .	10	less than 10
Colon bacillus 6-18 . . . . . less than 10	less than 10	less than 10
Typhoid bacillus . . . . . less than 10	less than 10	less than 10

The absorption tests were carried out by adding the bacilli from recent agar cultures to a 10 per cent. solution of the serum in a twenty-four-hour bouillon culture. The mixture was allowed to stand for twenty-four hours at about 22° C. It was found that the agglutinin in a simple dilution of serum when left at 37° C. rapidly deteriorated. Thus, in an extreme instance a serum positive at 1 to 1500, when diluted with bouillon or salt solution 1 to 25 and left at 37° C. for twenty-four hours, lost 30 to 40 per cent. of its strength; at 22° C. it lost at times 15 to 20 per cent. Left for three hours, the loss only was 5 to 10 per cent.





bodies is interesting both as showing the variation of the bacteria and as one means of adapting themselves to resist destruction, since the bacteria which ceased to be agglutinated or absorb agglutinin probably also were less affected by other antibodies. These changes are probably due to alteration in the external layer of the cells, as can be noted in those forming visible capsules such as pneumococci. It is possible that there is also less development of agglutinogens.

**Non-agglutinable Strains.**—Sometimes strains of microorganisms are obtained which possess all the other characteristics of a type and yet do not agglutinate. When grown on artificial media for some time they may gain the property. Organisms freshly obtained from the blood and tissues frequently agglutinate to a much less degree than after several transfers on media.

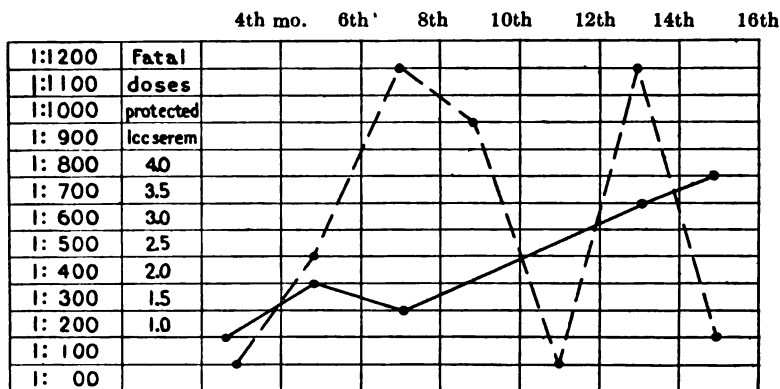


FIG. 77.—Relation of agglutinative to bactericidal power. Horse injected with culture of dysentery bacilli over a period of sixteen months.

— Agglutination index.

- - - Bactericidal index.

. Test dates.

**Relation between Agglutinating and Bactericidal Power.**—In spite of proof to the contrary good observers hold to the belief that there is some relation between the agglutinating and the bactericidal strength of a serum. The tests we carried out on the serum of a number of horses showed no such relation. In Fig. 77 are recorded a number of comparative tests during a period of sixteen months. While such an experiment shows no definite relation between agglutinin and immune bodies it must be remembered that these antibodies are the products of processes which are governed by similar laws, and so they have many points in common.

**Variation in the Agglutinating Strength of a Serum.**—There is usually a continued increase in the amount of agglutinin in the blood of an infected person from its first appearance at the end of from four to twenty-one days until convalescence and then a decrease. At times, however, there is a marked variation from day to day, so that it may be abundantly present one day and very slight in amount the next.

**Mode of Obtaining Serum from Blood or Blisters for Examination.**—

Fluid blood serum can easily be obtained in two ways: First, the serum may be obtained *directly from the blood*, thus: The tip of the finger or ear is pricked with a lancet-shaped needle, and the blood as it issues is allowed to fill by gravity a capillary tube having a central bulb. The ends of the tube are then sealed by heat or with melted wax, or candle-grease, and as the blood clots a few drops of serum separate. To obtain larger amounts of serum for a microscopic examination the blood is milked out from the puncture into a small homeopathic vial or test-tube. One cubic centimeter of blood can easily be collected in this way. The vial is then corked and placed on the ice to allow the serum to separate. As a rule one or two drops of serum are obtainable at the end of three or four hours. Second, the serum may be obtained *from blisters*. This gives more serum, but causes more or less delay. The method is as follows: A section of cantharides plaster, the size of a 5-cent piece, is applied to the skin at some spot on the chest or abdomen. A blister forms in from six to eighteen hours. This should be protected from injury by a vaccine shield or bunion plaster. The serum from the blister is collected in a capillary tube, the ends of which are then sealed. Several drops of the serum can easily be obtained from a blister so small that it is practically painless and harmless. The serum obtained is clear and admirably suited for the test. A piece of blotting paper soaked in strong ammonia when placed on the skin and covered by a watch-glass or strips of adhesive plaster will quickly raise a blister. A little vaselin should be smeared on the skin surrounding the blotting paper.

The whole blood may be used. It is dropped on glass or stiff paper, dried, and when it reaches the laboratory it is brought into solution.

**The Reaction.**—The reaction can be observed either macroscopically by sedimentation of the agglutinated clumps in a test-tube or on a glass slide, or microscopically in a hanging drop.

**The Macroscopic Tube Method of Estimating Amount of Agglutinins.**

—The tests are carried out with sets of test-tubes in racks as in the complement-fixation tests (see Chapter XI). Some prefer tapering tubes so that the sediment can be more easily estimated. As in complement-fixation tests, great care must be taken to have the test-tubes clean and free from chemicals. The bacterial suspension used, as in the microscopic test, must be standardized. The addition of the bacilli on a twenty-four-hour slanted agar tube to 150 c.c. of normal saline solution gives a good suspension. There must be a uniform cloudiness, not too heavy, with no signs of spontaneous agglutination.

A series of dilutions are made in which the suspensions of bacteria are of similar strength.

Tube.	Serum, c.c.	Salt solution, c.c.	Suspension of bacteria, c.c.	Final solution, c.c.
1	0.4	1.5	0.1	1 to 5
2	0.2	1.7	0.1	1 to 10
3	0.1	1.8	0.1	1 to 20
4	0.05	1.85	0.1	1 to 40
5	0.0	1.9	0.1	control

After thorough mixing, the tubes are allowed to stand in the incubator for one hour. They are then observed and the amount of flocculent precipitate noted. They are then placed in the ice-box at 10° or under, examined in twelve to twenty-four hours, and readings again made. Some varieties of bacteria agglutinate much more rapidly than others and for each, one must learn the proper time for reading the results. The tubes holding the greatest dilution of serum in which the fluid has cleared by the complete precipitation of all the bacteria shows the measure of the agglutinins. The control tube must be closely examined to note any sedimentation.

Heating the mixed serum solution and bacteria from thirty to sixty minutes to 55° C. gives good results for some varieties of bacteria.

**The Macroscopic Slide Method.**—This method allows a rapid diagnosis of colonies from plates inoculated with suspected material, such as feces, and can be employed in examinations for cholera, typhoid, paratyphoid and dysentery bacilli. A highly potent serum, whose specific and group agglutinating strength is known, should be used, or false positive results will be obtained; hence the method is not of much worth in clinical diagnosis of blood.

The method is as follows: A loopful of saline solution (as control) and one of the highly potent specific serum in low dilution are placed on one slide, and a sufficient amount of the suspected colony to give a slight turbidity is added to each. Flocculation begins in the serum almost at once if the organism tested is specific. A negative reaction is not exclusive as relatively inagglutinable strains may be infrequently encountered, though with highly potent serum there are always some evidences of a reaction. Colonies apparently typical but not distinctly agglutinable should be fished for further identification. Colonies giving a positive reaction should also be fished for verification unless experience has shown that the serum used does not give false positive results with allied types.

**Microscopic Reaction.**—If the reaction is observed through the microscope in a hanging drop, a formation of clumps is seen which, if it takes place rapidly, reveals the reaction almost completed at the first glance, that is, most of the bacilli are in loose clumps and nearly or altogether motionless (Figs. 78 and 81). Between the clumps are clear spaces containing few or no isolated bacilli. If the reaction is a little less complete a few bacilli may be found moving slowly between the clumps in an aimless way, while others attached to the clumps by one end are apparently trying to pull away, much as a fly caught in fly-paper struggles for freedom. If the agglutinating substances are present, but still less abundant, the reaction may be watched through the whole course of its development. Immediately after mixing the blood and the culture together it will be noticed that the bacilli move more slowly than before the addition of serum. Some of these soon cease all progressive movement, and it will be seen that they are gathering together in small groups of two or more, the individual bacilli being still somewhat separated from each other. Gradually they close up the spaces between

them, and clumps are formed. According to the completeness of the reaction, either all of the bacilli may finally become clumped and immobilized or only a small portion of them, the rest remaining freely motile, and those clumped may appear to be struggling for freedom. With blood containing a large amount of agglutinating substances all the gradations in the intensity of the reaction may be observed, from those shown in a marked and immediate reaction to those appearing in a late and indefinite one, by simply varying the proportion of blood added to the culture fluid. The reaction takes place more quickly when put in the incubator at a higher temperature (36° C.).



FIG. 78.—Grüber-Widal reaction. Bacilli gathered into one large and two small clumps, the few isolated bacteria being motionless or almost so.

**Pseudoreactions.**—If too concentrated a solution of dry blood from a healthy person is employed a picture is often obtained which may be mistaken for a reaction. Dissolved blood always shows a varying amount of detritus, partly in the form of fibrinous clumps, and prolonged microscopic examination of the mixture of dissolved blood with a culture fluid shows that the bacilli, inhibited by substances in the blood, often become more or less entangled in these clumps, and in the course of one-half to one hour very few isolated motile bacteria are seen. The fibrinous clumps alone, especially if examined with a poor light by a beginner, may be easily mistaken for clumps of bacilli. Again, the bacilli may be immobilized after remaining for one-half to two hours, by slight drying of the drop or by becoming attached to clumps of fibrin or other detritus. The reaction in disease is chiefly due to specific substances, but clumping and inhibition of movement similar in character may be caused by group agglutinins such as exist in normal horse and other serums. This is a very important fact to keep in mind.

In order to help the student to thoroughly understand what comprises a reaction Wilson prepared a set of drawings, which are here reproduced. The culture to be tested should be of about twenty hours' growth, either in bouillon or on agar. If on the latter a suspension

is made in broth or normal salt solution. A loopful of the fluid containing the bacteria is placed on the cover-glass, and to it an equal quantity of the desired serum dilution is added.

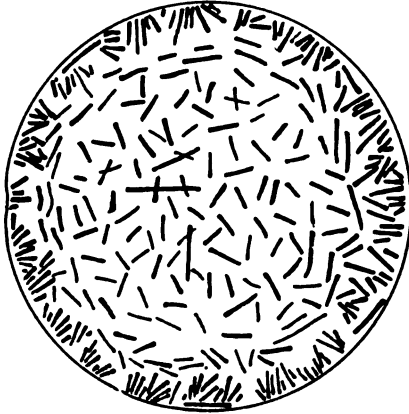


FIG. 79.—Microscopic field, showing the top of a hanging drop in a normal typhoid culture.

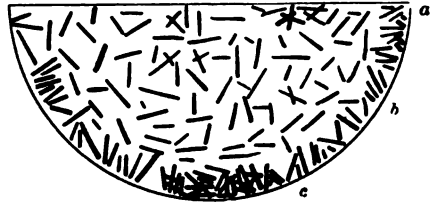


FIG. 80.—Microscopic field, showing a cross-section of the drop in Fig. 79.

In making the hanging drop to be examined it is necessary to have it of such a depth that it will show at least three focal planes, otherwise the examination will be incomplete and unsatisfactory. The moist chamber must be well sealed by vaselin so as to prevent drying, and kept at a temperature of at least 20° and not over 35° C.

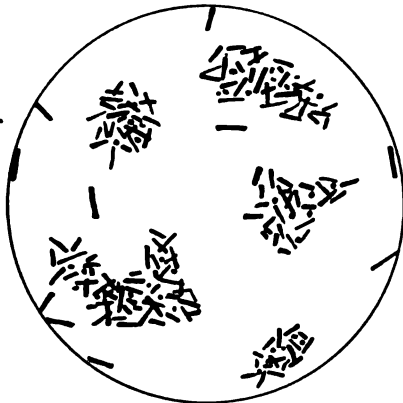


FIG. 81.—Microscopic field, showing the top of a drop with the typhoid reaction.



FIG. 82.—Microscopic field, showing a cross-section of the drop in Fig. 81.

Fig. 79 shows a microscopic field of the *top* of a hanging drop of a normal bouillon culture of typhoid bacilli. The culture is twenty

hours old and the organisms are freely motile. This represents the control drop used for comparison with the drop of the same culture to which has been added a little of the blood of a person suspected to have typhoid. Note these points in Fig. 79; the organisms are evenly distributed throughout the field, except at the edge of the drop, where they are gathered in great numbers; they show great activity here, seemingly trying to crowd to the very edge. This attraction is probably due to the action exerted on the organisms by the oxygen in the air, which naturally exerts positive chemotaxis on all aerobic organisms.

Fig. 80 shows a *cross-section* of the drop represented in Fig. 79, and it will be noticed that the bacilli are evenly distributed throughout the drop, except at one place in the focal plane *a*, and again in the focal plane *c*.

It sometimes happens that there is a substance adhering to a supposedly clean cover-glass which attracts the bacilli to that point, where they appear as fairly well-defined clumps, more or less like the true clumps due to the agglutinating substance in typhoid blood. The increase in organisms at the bottom of the drop in the focal plane *c* is easily accounted for by the fact that gravity naturally carries the dead and non-motile organisms to the bottom, these frequently assuming the character of clumps.

If a field can be found in any focal plane of the hanging drop free from clumps, one can be quite sure that any clumping present is not due to any agglutinating substance which necessarily will affect organisms in every focal plane.

Fig. 81 shows the microscopic appearance of the *top* of a drop where the reaction is present. Notice first that the organisms have been drawn together in groups and that the individuals of each group appear to be loosely held together. Viewed under the microscope these clumps are practically quiescent, there being very little movement either of the individual organisms or of the clump as a whole. The edge of the drop is practically free from organisms, showing that the air no longer exerts any influence on them.

Fig. 82 shows a *cross-section* of the hanging drop shown in Fig. 81. The clumps are evenly distributed throughout the drop, with perhaps some increase in the numbers and compactness of the clumps at the bottom.

Fig. 83 shows the microscopic appearance of the *top* of a hanging drop of a bouillon culture to which has been added some blood of a patient suffering from a febrile condition not caused by typhoid infection, but which exerts a slight non-specific influence on the typhoid organisms. It will be seen that the reaction is incomplete and that there are many organisms at the edge of the drop. The air exerts the same influence on the bacilli that it did before the addition of the blood. Note the character of the clumps, generally small and compact at the centre, with the bacilli at the edge of the clump, usually attached by one end only.

Very frequently these clumps have the appearance of being built up around a piece of detritus present in the clump. All the organisms comprising the clump seem to have retained part, at least, of their motility, those on the edges being particularly motile, so far as their free ends are concerned.

When motility is very much inhibited these clumps have a peculiar trembling movement, which is like the molecular movement described by Brown.

Fig. 84 shows a cross-section of the drop represented in Fig. 83. Note the same character of the clumps in every focal plane: the large number of motile bacilli and the number attracted to the edge of the drop by the air.



FIG. 83.—Microscopic field, showing the top of a drop of culture with reaction not due to typhoid.

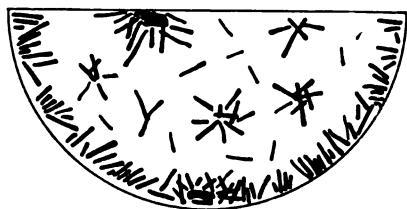


FIG. 84.—Microscopic field, showing a cross-section of Fig. 83.

**Comparison of Tube and Microscopic Slide Methods.**—The reaction is the same in both and one is as reliable as the other. The ice-box readings are apt to be higher than those obtained by microscopic examination. For diagnostic examinations where haste is necessary and small amounts of serum are available, as in typhoid fever, the microscopic method is preferred. When a delay of twenty-four hours is no handicap and the serum is abundant as in tests for glanders in horses the macroscopic tube test is chosen. For the identification of bacteria the macroscopic test is generally used. Dead cultures are more frequently used in the macroscopic method because the motility is of no importance.

As noted above, the growth of bacteria in fresh blood containing agglutinins inhibits the development of agglutinable substance in bacteria or causes them to produce substances which prevent the union of agglutinin with them. Bacteria should therefore not be grown on serum media when they are to be used in agglutination tests. Even the addition of ascitic fluid to broth has some effect.



**THE PHENOMENON OF PRECIPITATION.**

**Precipitin.**—The injection into the animal body of the protein substances of bacteria or of other cells was found to stimulate the development of agglutinin almost as much as the injection of the unaltered cell. Kraus, in 1897, found that when a little immune serum was added to the bacteria-free filtrate of a culture of the organism used to produce the immunization there occurred a precipitate. This he designated as the "precipitin reaction." This same reaction took place between the serum of an animal and various protein substances, such as white of egg, blood serum, or milk, with which the animal had been injected. That part of the protein which produces the precipitin is called precipitinogen (antigen). Precipitins in their development, their resistance to heat and chemicals, and in their specific and non-specific forms are similar to agglutinins. The specificity of precipitins is, like that of the agglutinins, not absolute. Group precipitins act upon similar chemical substances derived from cells having very different characteristics. The precipitin test is usually employed in blood identification and in testing sera and tissue extracts rather than bacterial filtrates. As the reaction depends on the formation of a precipitate it is important that the solution of serum and antigen be absolutely clear before being placed together.

With bacterial antigens the test is carried out by placing a constant amount of the precipitinogen in a row of test-tubes and adding decreasing amounts of immune serum (precipitin). A series of control tubes of each serum should be observed. The tubes are placed in the incubator. With potent serums precipitation becomes visible after one-half to two hours.

With serum or tissue extracts as antigens a constant amount of antiserum is placed in a series of tubes and then equal parts of dilutions of the antigen, the dilutions ranging from 1 to 10 to 1 to 5000 or beyond. A very highly potent serum will give a precipitate with even dilutions of 1 to 10,000 or beyond.

**Production of Precipitating Serums.**—Animals, usually rabbits, are injected with bacterial suspensions heated to 60° C. or with the filtered bacterial extracts obtained by emulsifying bacteria grown on agar in salt solution and shaking in a shaking machine for forty-eight hours. The injections are made just as in the production of agglutinins, but a longer period of immunization is necessary to produce a highly potent precipitating serum. Sera or protein extracts are injected intravenously in amounts of from 1 to 3 c.c. at 5-6 day intervals. Three or four injections usually suffice. Some rabbits do not respond well so that several should be injected.

## CHAPTER XIII.

### OPSONINS. OPSONIC INDEX. LEUKOCYTE EXTRACT.

#### OPSONINS.

WE find that phagocytosis is most marked when the disease is on the decline or the infection mild, but is usually absent in rapidly increasing infection. This would seem to indicate that the course of the infection is often already determined before the leukocytes become massed at the point of its entrance. The first determining influence is given by the condition of the tissues and the amount of bactericidal substances contained in them, and then, later, in cases where the bacteria have been checked, comes the additional help of the leukocytes. If the tissues are wholly free from bactericidal and sensitizing substances, neither they nor the leukocytes, nor both combined, can prevent the increase. The simple engulfing by the cells of bacteria is not necessarily a destructive process. (See also p. 164).

The interest in the subject of the opsonins is largely due to the investigations and influence of Wright. We should, however, recognize the important earlier work of others. Denys and Leclef had previously shown that in the case of rabbits immunized against streptococci, the increased phagocytosis was due to an alteration in the serum and not to changes induced in the leukocytes. They demonstrated that the leukocytes of the immunized animal when placed in normal serum showed no greater phagocytic activity than normal leukocytes did, and that therefore the substances in the serum favoring phagocytosis united with the bacteria. Wright and Douglass showed definitely that phagocytosis of bacteria in normal serum depends upon a special substance in the serum which becomes fixed to the bacteria and prepares them for phagocytes. They called this substance opsonin. Neufeld and Rimpau discovered the same point independently. Wright dealt mostly with normal serum, while Neufeld used serum from immunized animals.

Wright originated the idea of estimating the changes in the opsonic power of the blood for the purpose of guiding the use of vaccines in the treatment of bacterial infections. Thus he states:

"I have found that there exists in the serum of the successfully inoculated patient an increase of opsonin. This is a substance which lends itself to very accurate measurements by a modification of Leishman's method. By the aid of this method the patient's progress or regress can be very accurately followed."

Where vaccines are injected, Wright states, there "supervenes a negative phase where there is a diminished content in protective substances. This is succeeded by a positive phase. This inflowing wave of protective substances rapidly flows out again, but leaves behind in the blood a more or less permanently increased content of protective substances. When a small dose of vaccine is given the negative phase may hardly appear, but the positive phase may be correspondingly diminished. Where an unduly large dose of vaccine is

inoculated the negative phase is prolonged and much attenuated. The positive phase may in such a case make default.

"It will be obvious that, if we, in the case of a patient who is already the subject of a bacterial invasion, produce by the injection of an excessive dose of vaccine a prolonged and well-marked negative phase, we may, instead of benefiting the patient, bring about conditions which will enable the bacteria to run riot in his system.

"Now consideration will show that we may obtain, according as we choose our time and our dose wisely or unwisely, either a cumulative effect in the direction of a positive phase or a cumulative effect in the direction of a negative phase. We may in other cases, by the agency of two or more successive inoculations, raise the patient by successive steps to a higher level of immunity, or, as the case may be, bring down by successive steps to a lower level. We can select the appropriate time and dose with certainty only by examining the blood and measuring its content in protective substances in each case before reinoculating."

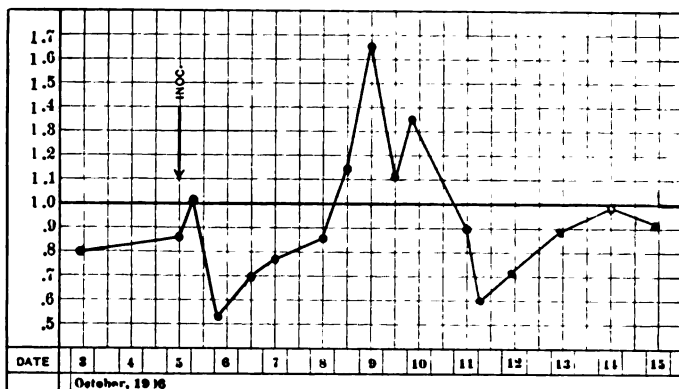


FIG. 85.—Opsonic curve showing the slight immediate rise and the later negative and positive phases following inoculation. The changes here are more regular than generally occurs.

An immense amount of investigation has revealed the fact that an estimation of the opsonin cannot be obtained accurately enough in single tests to be a safe guide to be used in diagnosis or treatment unless the variation from the normal is exceptionally great, and that the opsonic content is not alone a safe guide for the measure of the total antibodies in the blood.

### THE OPSONIC INDEX.

**Technic.**—Wright's technic of measuring the opsonic power is a slight modification of the Leishman method and is as follows: An emulsion of fresh human leukocytes is made by dropping twenty drops of blood from a finger prick into 20 c.c. normal salt solution containing 1 per cent. sodium citrate. The mixture is centrifuged, the supernatant clear fluid removed, and the upper layers of the sedimented blood cells transferred by means of a fine pipette to 10 c.c. normal salt solution. After centrifuging this second mixture the supernatant

fluid is pipetted off and the remaining suspension used for the opsonic tests. Such a "leukocyte emulsion," of course, contains a mixture of leukocytes and of red blood cells; the proportion of leukocytes, however, is much greater than in the original blood. The bacterial emulsion

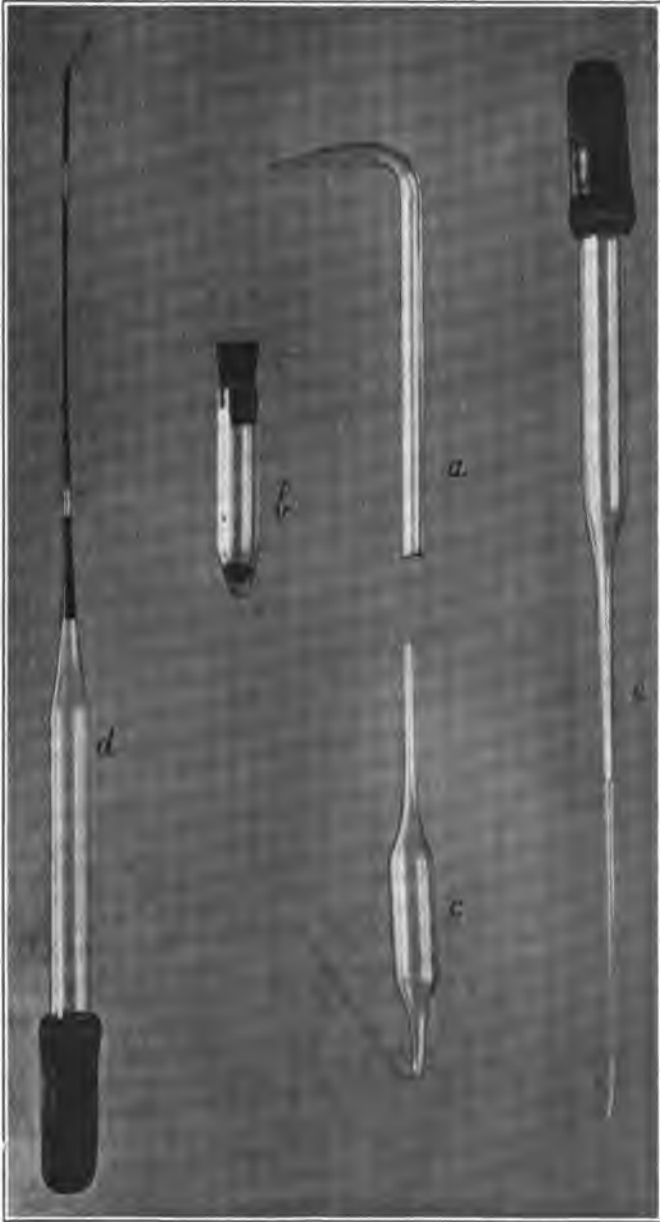


FIG. 86.—Opsonic outfit.

is prepared by gently rubbing a little of the culture to be tested in salt solution (0.85 to 1.2 per cent.). When thoroughly mixed the fluid is centrifuged for a few minutes so as to remove any clumps. The emulsion should be so thick that in a trial test the leukocytes take up about five apiece on the average.

One volume of the leukocytes is mixed with one volume of the bacterial emulsion and with one volume of the serum to be tested. This is best accomplished by means of a pipette, suggested by Wright, whose end has been drawn out into a capillary tube several inches in length. With a mark made about an inch from the end it is easy to suck up an equal volume of each of the fluids, allowing a small air-bubble to intervene between each volume. All three are now expelled on a slide and thoroughly mixed by drawing back and forth into the pipette. Then the mixture is sucked into the pipette, the end sealed, and the whole put into the incubator at 37° C. (Fig 86, *d* and *e*). The identical test is made using a normal serum in place of the serum to be tested. Both tubes are allowed to incubate fifteen minutes and then the end of the tube is broken off, a large drop mounted on a clean slide the surface of which was previously roughened by emery-paper, and a spread made with a second slide as in ordinary blood work, only a little thicker and using no force whatever. After drying in the air the smears are stained without previous fixation either with a 1 per cent. aqueous solution of methylene blue or some other suitable stain. The degree of phagocytosis is then determined in each by counting a consecutive series of fifty or one hundred leukocytes and finding the average number of bacteria ingested per leukocyte. This number for the serum to be tested is divided by the number obtained with the normal serum (obtained by pooling the serum of healthy persons) and the result is regarded as the *opsonic index* of the serum in question. Thus, if the tubercle bacilli, sensitized by a patient's blood, are taken up by the leukocytes to the average number of three per leukocyte, and bacilli from the same emulsion sensitized by normal blood are taken up by leukocytes to the average of five, then the index will be three-fifths of one, or 0.6. Phagocytic index (normal pooled blood), 5; phagocytic index (patient's serum), 3; opsonic index,  $\frac{3}{5}$  or 0.6. In this case the index would indicate a deficiency in opsonins. The presence of a high opsonic index Wright regards as indicative of increased resistance. He further states that the fluctuation of the opsonic index in normal healthy individuals is not more than from 0.8 to 1.2, and that an index below 0.8 is therefore almost diagnostic of the presence of an infection with the organism tested.

A number of workers use the washed whole blood. This saves in original labor, but makes the search for a suitable number of leukocytes more difficult.

*The Dilution or Extinction Method* recommended by Dean and by Klein. The degree of dilution of the serum necessary for the extinction of its opsonic index is determined; that is, the serum to be tested is diluted until a dilution is found which shows the same small amount

of phagocytosis shown in preparations in which no serum is used, namely, an index below 0.5. Klein claims that results by this method are more accurate than by the method of Wright. The method is too tedious for practical use in routine work.

*Combined Method.*—Simon estimates the percentage of phagocytizing cells in the mixture containing the serum to be tested and compares this with the mixtures containing normal serum. He does this not only with the undiluted serum, but also in dilution of 1 to 10 or 1 to 100 in salt solution.

Most workers now agree that the use of the opsonic index is limited to experimental investigations. The reasons for this opinion follow:

THE ACCURACY WITH WHICH THE OPSONIC POWER OF THE BLOOD CAN BE DETERMINED BY WRIGHT'S METHOD.—An examination of any slide will show that the different leukocytes vary in their size and in their content of bacteria. This is due partly to variation in phagocytic activity, and partly to the interference of the red blood cells, which are present in great numbers in the emulsion and separate the bacteria in different degrees from the white cells. These and other reasons bring it about that the different leukocytes vary greatly in the number of bacteria they take up and in their distribution on the slide. Partly to overcome this, large numbers of leukocytes are counted. Beyond one hundred, or at most one hundred and fifty, the increase of accuracy hardly compensates for the extra labor. The following table shows the difference between counting larger or smaller numbers of cells in five opsonic tests as determined by counting different numbers of cells in one specimen:

OPSONIC INDEX ESTIMATIONS IN FIVE BLOOD SPECIMENS.

Cells counted.		Average number of bacteria in each leukocyte.				
50	. . . . .	1.18	1.88	1.34	1.42	1.90
100	. . . . .	1.22	1.78	1.24	1.42	1.59
150	. . . . .	1.18	1.62	1.22	1.44	1.50
200	. . . . .	1.18	1.51	1.22	1.46	1.37
600	. . . . .	1.28	1.62	1.23	1.36	1.36
1,200	. . . . .	1.34	1.44	1.25	1.30	1.42

It is noticed that the variation between the average cell count obtained from fifty cells and larger numbers is much greater than between that obtained at from one hundred or one hundred and fifty.

It is necessary to have the counts that are compared all counted by the same person, as each individual has a somewhat different method and may average higher or lower for all counts than any other person.

When two specimens of blood are tested not only the inaccuracy of counting due to the different arrangement of the unequally filled cells on the slides to be counted is met, but the fact that in making the test the conditions are not similar, for in different mixtures slightly different proportions of leukocytes, bacteria, and red cells will always be mixed together. If smears from a series of tubes of the same blood are compared with a series of smears from one of the tubes, the former will always show the greater variation.

This variation is much greater than most examiners believe. North has collected a series of tests carried out in nearly all the important laboratories in the Eastern United States that are working upon opsonins. The results recorded prove absolutely that while an average counting error of only about 10 per cent. is present, there may be an exceptional error of at least 100 per cent., and one of at least 20 per cent. may be expected once in about every ten determinations.

The following is a fair average of the correctness of routine tests by experienced workers.

ABSOLUTE COUNT OF BACTERIA IN ONE HUNDRED LEUKOCYTES.

Blood specimen A.			Blood specimen B.			Blood specimen C.		
Tube 1 . . .	156		Tube 1 . . .	142		Tube 1 . . .	89	
Tube 2 . . .	168		Tube 2 . . .	182		Tube 2 . . .	102	
Tube 3 . . .	172		Tube 3 . . .	188		Tube 3 . . .	121	

This error, which occurs because of the technic, applies not only to the examination of the specimen of blood, but also to the measure we employ to estimate the amount of opsonins. As these are not stable, we cannot have a standardized solution, as we do with anti-toxins. We must therefore determine our measure afresh in each test, taking for this purpose a supposedly normal blood. Wright, from a great many tests, has determined that the opsonic power of the blood in non-infected persons for tubercle bacilli does not vary, as a rule, more than 10 per cent. above or below the average power of healthy blood. For staphylococci there is more variation. It is found also that many things besides infection decrease the amount of opsonins in the blood. Hemorrhage, fatigue, starvation, and other influences which lower the resistance of the body have this effect.

Wright gets this measure as uniform as possible by determining the average opsonic strength of five supposedly healthy persons at the time of each test. If any one of these five is considerably below or above others it is omitted for the day. The measure so obtained will probably vary about 5 per cent. from day to day, though seldom getting far away from what we might call the absolute normal. The following results were obtained by us from examining at one test a number of supposedly normal persons against staphylococci. The variation is greater than in Wright's reports.

OPSONIC COUNTS IN TEST OF TWENTY-ONE NORMAL SERA WITH STOCK STAPHYLOCOCCUS CULTURE.

1 . . . . .	4.13	8 . . . . .	3.82	15 . . . . .	9.09
2 . . . . .	2.93	9 . . . . .	3.95	16 . . . . .	5.17
3 . . . . .	2.78	10 . . . . .	3.98	17 . . . . .	4.04
4 . . . . .	4.37	11 . . . . .	4.27	18 . . . . .	3.82
5 . . . . .	3.58	12 . . . . .	3.69	19 . . . . .	4.00
6 . . . . .	2.90	13 . . . . .	3.80	20 . . . . .	3.79
7 . . . . .	3.56	14 . . . . .	3.59	21 . . . . .	3.44

THE INFLUENCE UPON THE OPSONIC TEST OF THE SPECIFIC DIFFERENCES BETWEEN STRAINS OF A SINGLE SPECIES.—The general practice

in laboratories is to use stock cultures of tubercle bacilli, staphylococci, and other bacteria for the opsonic tests. To obtain a culture from a case may be at first impossible and, if successful, causes a delay of at least one or two days. The culture when obtained may also, as is frequently the case with pneumococci and streptococci, fail to opsonize readily.

These and other reasons tend to establish the use of laboratory stock cultures, and yet we must acknowledge that when we test the amount of opsonins by both the stock and fresh cultures a marked difference sometimes develops.

**THE LEUKOCYTES TO BE EMPLOYED.**—To many it seems a matter of indifference whether one person's leukocytes or another's are used, but our experience agrees with that of others that the leukocytes from different persons not only vary in their activity, but also in their selective action, and that the index is not the same when obtained with one person's leukocytes as with another's.

**THE INFLUENCE OF THE STRENGTH OF THE BACTERIAL EMULSION.**—The more abundant the bacteria the greater will be the number taken up by the leukocytes. It is very important therefore that the tests be made with the same strength of emulsion.

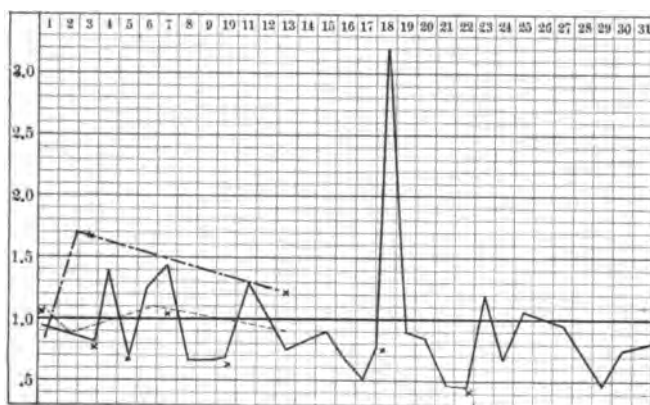


FIG. 87.—x = injection of vaccine. Three types of lines = 3 cases.

**THE OPSONIC VARIATION DURING TREATMENT BY INOCULATIONS.**—Wright lays stress on the considerable uniformity of the degree and persistence of development of opsonins after inoculation. We have found in a small percentage of cases typical increases and decreases, as seen in Fig. 85, but in the majority of those inoculated there has been great irregularity. Frequently the negative phase does not occur or at least it is not detected. The following chart for three staphylococcus cases illustrates this (Fig. 87):

**THE VARIATION FROM DAY TO DAY IN THE AMOUNT OF OPSONINS IN SUPPOSEDLY HEALTHY PERSONS.**—It has already been noted that in getting our measure we test a number of persons and exclude the blood



of those which varies greatly from the average. We are so in the habit of seeing the index of normal blood placed at unity because it is each day the measure of comparison that even investigators are apt to think of the indices of normal persons as being unchanged from day to day. This is not the fact. A glance at the next chart (Fig. 88), in which four cases of tuberculosis are charted together with two normal persons, shows that the variation is only slightly greater in infected than in normal cases. If one normal person is charted against another for several weeks, marked differences will usually appear. The indices of the twenty-one normal cases tested against staphylococci (page 222) illustrate this variation in the amount of opsonins in normal blood.

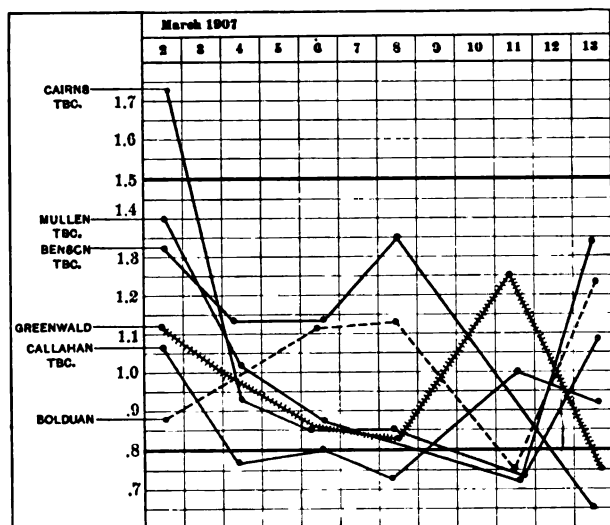


FIG. 88.—Dotted and crossed lines, normal persons. Continuous lines, tuberculosis.

**THE OPSONIC INDEX CANNOT BE KNOWN AT THE TIME THE TREATMENT IS GIVEN.**—Most of those who have not carried out inoculations under the guide of the opsonic tests think that the vaccinator is guided at the moment of injection by his knowledge of the opsonic power of the blood at the time. A moment's thought reveals that this is an absolute impossibility. In fact, except under very unusual conditions, it is impossible to have the test of the opsonic power reported within twenty-four hours, and in the treatment of the poor in out-patient practice longer intervals usually elapse, so that the treatment is given on a test made either the day before or, more often, on from three to seven days before. As can be seen by the three curves in Fig. 86, which are quite as uniform as the average, it is impossible to judge what the index is at any moment by looking at the indices of blood taken from one to seven days previously.

The other methods devised to get more accurate information upon the opsonic contents of the blood are more time-consuming. For

experimental work they have advantages, but for practical use in governing the dosage of vaccines they have most of the drawbacks of Wright's method.

**THE DIAGNOSTIC VALUE OF OPSONINS.**—The presence of a great excess or deficiency of opsonins for a microorganism, or of marked variation in the index after massage or exercise, has been thought by some to indicate the type of infection. Extreme caution should be used in making such an application of the index determinations.

**OPSONIN DEFICIENT IN CEREBROSPINAL FLUID AND IN EXUDATES.**—Opie has shown that exudates produced by injecting microorganisms usually have little or no opsonin for the variety injected or for other varieties. Hektoen has showed that opsonins, like other antibodies, are almost absent in the spinal fluid. McKenzie and Martin showed that in a case of cerebrospinal meningitis the spinal fluid showed no immune bodies, while the blood contained them in abundance.

### THE THERAPEUTIC VALUE OF LEUKOCYTE EXTRACT IN INFECTIONS.

Petterson found sera in which abundant leukocytes were present to become more bactericidal. He attributed this change to substances given up by the leukocytes. He found that the leukocytes injected into the peritoneum of dogs gave some protection against anthrax infection but none against typhoid infection. He considered the leukocytes probably acted indirectly in stimulating the flow of complement and antibodies to the peritoneal cavity.<sup>1</sup> He makes this extract as follows:

The leukocytes are obtained by double pleural inoculations with aleuronat into the animal (rabbit, dog). The amount of leukocyte-filled fluid obtained after twenty-four hours from rabbits has usually been from 30 to 60 c.c. This is immediately centrifugalized, the serum poured off, and the extracting fluid (distilled water) added in amounts about equal to the fluid poured off. The cells are then thoroughly emulsified in the distilled water, allowed to stand for eight hours at 37.5° C., and then at ice-box temperature until used. Varying amounts of the entire fluid (after shaking) are inoculated. Hiss's animal experiments were made on rabbits and guinea-pigs infected with staphylococcus, streptococcus, pneumococcus, typhoid bacillus, or meningococcus. Hiss stated that animals suffering from severe septicemias and poisonings following intravenous injection of any one of the above organisms have shown the beneficial effect of treatments with extracts of leukocytes, and have, in many instances, survived infections fatal to the control animals in thirty-six hours, even when treatment has been delayed as late as twenty-four hours. Zinsser has carefully studied the nature of the substances extracted from leukocytes. He finds they contain no complement, are not destroyed by heating to 56° C., and are no more abundant in cells derived from immunized than from normal rabbits.

<sup>1</sup> Hiss tried out the extract in a number of human cases as well as in infected animals.

The Hiss leukocytic extract has now been used by a number of observers for some six years. Cases of pneumonia, erysipelas, septicemia, and some other infections have been treated. It is difficult to determine just what the value of the treatment is. The effects are probably similar to those obtained by the use of other more specific proteins. No harmful results have been noticed. In a certain number of cases the temperature and symptoms have bettered in a way which seemed clearly to indicate that the extract had done good. In other cases no results whatever were apparent. In our personal experience we have not seen marked results. The extract is usually given subcutaneously in 10 c.c. doses every four to six hours. As high as 500 c.c. have been given in some cases.

Zinsser in his book on *Infection and Resistance* believes the use of the extract should be confined to infections in which the process is localized.

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## CHAPTER XIV.

### PROTEIN HYPERSENSITIVENESS OR ANAPHYLAXIS.

PERHAPS the most important recent addition to our scientific knowledge has been the development of our conception of the fact and meaning of protein hypersensitiveness. Magendie was probably the first to record observations on this subject. He noted (1839) that rabbits which were injected intravenously for the first time with egg albumin showed no untoward effects, but that if after a lapse of time a second injection was given, serious and perhaps fatal results followed.

In 1894 Flexner noted the same results in animals receiving a second injection of dog serum. In 1902 Richet, Portier and Hericourt noted that when a moderately poisonous protein such as actinocongestin was injected for the first time in dogs 0.05 gram per kilo caused sickness but not death. After the lapse of some days and full recovery a dose one-twentieth as large not only caused sickness but also death. The previous injection had, apparently, instead of stimulating a protective antibody, produced a ferment which rendered the animal more susceptible. The fact of this lack of protection caused Richet to give the term anaphylaxis to this condition. This term has been applied to this condition of sensitization even though the protein is entirely harmless in its first dose. Arthus showed that in animals sensitized to horse serum a later injection given subcutaneously produced striking cutaneous reactions ranging from infiltration to gangrene. Theobald Smith, in 1905, studied and reported the reaction in guinea-pigs after injections of horse serum, and Rosenau and Anderson noted the fact that the young of anaphylactic mothers inherited the same protein sensitiveness. They found also that in guinea-pigs about ten days elapsed before a dose of serum caused sensitization, and that the reaction is quantitatively specific and is extremely delicate. An injection of one-millionth of a cubic centimeter may sensitize animals and this condition persist for a year or more. Not only animal and vegetable proteins but also bacterial proteins have the property of producing this hypersusceptibility. Vaughan and others explain the poisoning of the second injection as follows: The introduction of a foreign protein into the tissues or blood of an animal develops in that animal a proteolytic ferment which is specific for the protein injected or for one very closely allied to it. The specific ferment remains as a zymogen in the cells of the animal and in its blood and tissue fluids, and is activated when the same protein is again injected.

All proteins contain a toxic and a non-toxic portion; the first is not specific, while the second is. The first injection of the protein produces no symptoms because there is no proteolytic ferment present. The

second injection, if sufficient time has elapsed for the formation of the ferment, causes symptoms because the specific ferment having been produced, the protein when re-injected is split up into poisonous and non-poisonous portions, and the former causes symptoms of intoxication or anaphylaxis. Those changes occur not only in the plasma, but also in the cells, and the symptoms depend on whether the second injection was made locally or into the general blood current. The second injection causes no symptoms unless, as in all other immunity reactions time enough is allowed to elapse for the cells to assimilate the proteins and for specific ferments to be produced.

Gay and Southard applied the term anaphylactin to this ferment. Friedberger calls the poisonous part of the split protein anaphylatoxin.

Jobling and Peterson believe from their experiments that the poisoning results, partly at least, from the fact that the bacteria attack and make inactive antiferments in the blood and thus allow ferments of the blood previously kept from acting on the serum to act. There may be not only split products of the protein injected but also of the serum of the animal receiving the second injection.

*Amount Required to Sensitize.*—Very small amounts such as 0.0001 c.c. of horse serum sensitizes an animal by whatever way inoculated.

*Incubation.*—This varies in different animals. In man it is usually seven to twelve days. It gradually reaches its maximum and lasts for four months to a year or more. When the reaction is present after much longer periods there has probably intervened a later sensitization, just as must have happened when an anaphylactic shock occurs in persons after a primary serum injection.

*Quantity of Foreign Protein Required to Produce Symptoms.*—This is much larger than the amount which sensitizes. Much smaller amounts produce symptoms when given intravenously than when given subcutaneously. Thus, in a guinea-pig, 0.01 c.c. of serum intravenously has as much effect as 0.5 c.c. subcutaneously.

The following varieties of proteins produce antiferments:

1. Animal proteins in solution: Foreign serum, hemoglobin, milk albumin, egg albumen, etc.
2. Cellular animal proteins: Red blood cells, leukocytes, cells of organs and tumors.
3. Extracts of vegetable proteins: From bacteria (tuberculin, mallein), seeds, etc.
4. Cellular vegetable proteins: Living or dead bacteria and yeasts.

*Specificity.*—When an animal is sensitized with one protein it reacts most strongly to one of the same chemical structure, but also slightly to others nearly allied; thus a guinea-pig sensitized to egg albumen of the hen reacts slightly to egg albumen of the duck. This is similar to the group reaction of agglutinins, precipitins, etc.

*Antianaphylaxis.*—Besredka and Steinhardt noted that animals recovering from a second injection of serum are for a time not sensitive to another injection. This condition lasts for a variable time.

The effect of protein sensitization may be manifest in many ways.

Thus von Pirquet and Schick, in 1905, described the symptoms which frequently follow in man after an injection of horse serum. These symptoms follow almost as frequently primary as secondary injections, but the time of their appearance is late with the primary injections and early with the secondary ones. The probable explanation is that after the injection of the first serum the cells gradually assimilate it and in a few days develop the specific proteolytic ferment. This acts upon the portion of the serum not yet assimilated and, splitting it into the poisonous and non-poisonous portions, the toxic symptoms of fever, rash, etc., develop. The development of the symptoms in infectious diseases is attributed by Vaughan and others to this same fact of protein cleavage, as in the case of serum injections.

**Symptoms of Serum Sickness.**—Under this name we now include the various clinical manifestations following the injection of horse serum into man. The principal symptoms of this disease are a period of incubation varying from three to thirteen days, fever, skin eruptions, swelling of the lymph glands, leukemia, joint symptoms, edema, and albuminuria. The term "serum sickness" was first used by von Pirquet and Schick, from whose excellent monograph the following data are chiefly taken.

In 1874 Dallera reported that urticarial eruptions may follow the transfusion of blood. In the year 1894 the use of diphtheria antitoxin introduced the widespread practice of injecting horse serum. In the same year several cases were reported in which these injections were followed by various skin manifestations, mostly of an urticarial character. Following these came a great mass of evidence which made it clear that following the injection of antidiphtheritic serum these sequelæ were usually comparatively harmless.

**DUE TO SERUM AS SUCH.**—Heubner, in 1894, and von Bokay, somewhat later, expressed the opinion that these manifestations were due to other properties than the antitoxin in the serum, and this has proved to be the case. It has also been shown that the skin eruptions and other symptoms follow in a considerable degree according to the amount of serum injected, and this has led to attempts to eliminate the non-antitoxic portion of the serum as much as possible. The serum reaction has been studied by many investigators, but is not yet fully understood.

**Danger.**—About 1 in 20,000 primary injections of diphtheria antitoxin sera causes the immediate appearance of anaphylactic shock, in which symptoms of respiratory embarrassment and convulsions develop. About 1 in 50,000 of these cases ends fatally. Friedberger and Hartock have shown that with the occurrence of anaphylactic symptoms there is a diminution of complement.

**Anaphylactic Shock in Man.**—It is probable that man cannot be sensitized in the same way as the guinea-pig, the most susceptible of the laboratory animals. Children have, in numerous instances, been injected with antidiphtheritic horse serum at short and long intervals without, so far as we are aware, causing severe symptoms. Certain serums—for

example, the antitubercle serum of Maragliano—are habitually used by giving injections at intervals of days or weeks. The rare fatal cases so far reported have all followed primary injections.

There is, in our experience, no reason to avoid a second immunizing injection of serum when it is really indicated. A subcutaneous injection in man comparable to the amount required to produce sickness in a guinea-pig would be over 200 c.c. We should hesitate, however, to give a large intravenous injection in a sensitized child. Banzhaf and Famulener have shown that chloral in large doses will prevent sickness in sensitized guinea-pigs.

### **FERMENTS AND ANTIFERMENTS AND THEIR RELATION TO SOME PHASES OF ANAPHYLAXIS.**

THE hypothesis advanced by Vaughan (1906) that intoxications accompanying infections are produced by toxins liberated by specific proteolytic ferments acting on bacteria, and later by Abderhalden (1912) that the blood in pregnancy contains a specific ferment which is able to digest placental tissue, has called forth a series of investigations which appear to throw considerable light on some immunity problems. The specificity of these ferments has, in recent years, been seriously questioned, the consensus of opinion being that these ferments are non-specific in character and that their action depends largely on the anti-ferment content of the blood and tissues. Thus the protein-split products which are often produced when placental tissue is incubated with serum of pregnancy or when bacteria are incubated with their homologous antisera are not due to the splitting of placental or bacterial proteins but rather to the digestion of the serum proteins. The placental tissue and bacteria absorb the antiferments, removing thereby the check normally exerted on the ferments. Substances such as kaolin, agar and starch when incubated with serum will also absorb antiferments and digestion of serum proteins will take place under these conditions. The work of Jobling and his co-workers particularly deserves mention in this connection. These investigators found that antiferments may be easily extracted with ether and chloroform. On the other hand, lipid substances and soaps they found to inhibit proteolytic action.

**Serotoxins, Anaphylaxis and Anaphylotoxins.**—Serotoxin is a term applied by Jobling and co-workers to serum which is rendered toxic by removing the antiferments. The toxicity they believe to be due to the protein cleavage products brought about by the action of the proteolytic ferments. Guinea-pig serum treated with chloroform to remove the antiferments is toxic for other guinea-pigs in doses of 0.003 c.c. to 0.01 c.c. per gram weight. Of interest in this connection is the fact that the symptoms and postmortem findings in such cases are similar to those observed in anaphylaxis.<sup>1</sup> Furthermore, guinea-pigs sensitized with horse serum, on receiving injections of serum lipoids (antiferments) twenty-four hours previous to the second and intoxicating dose were

<sup>1</sup> These findings have recently been seriously questioned by Smith, H. R., *Jour. Lab. Clin. Med.*, 1916, i, 584.

able to withstand twice the minimal lethal dose of the antigen. Judging from these findings, anaphylaxis appears to be largely a disturbance in the ferment and antiferment balance.

The similarity of the results these investigators obtained with serotoxins to those obtained with anaphylatoxins also deserves mention in this connection. The toxicity of anaphylatoxin is apparently not due to the toxins derived from the bacteria as is believed by many authors, but rather to the fact that the bacteria absorb the antiferments, exposing thereby the serum proteins to the action of ferments and to the production of toxic-split products. If, for instance, the bacteria are first treated with lipoids and then incubated with serum, anaphylatoxin is not obtained, owing presumably to the fact that the bacteria being saturated with oils are unable to take up the lipoids of the serum.

The marked resistance of some bacteria, dead or alive, to ferment action is also explained by the inhibitory action of lipoids on tryptic digestion. The limiting membrane of bacteria is believed to be lipoidal in character. This membrane is thus able to exert its antiferment powers when exposed to ferment action. Thus, in the case of tubercle bacilli, the amount of digestion by trypsin was found to be inversely proportional to the amount of lipoids they contained. Organisms partially extracted containing 7 per cent. of lipoids gave 57 per cent. of digestion while organisms containing 31 per cent. of lipoids gave only 23 per cent. of digestion.

An interesting point brought out by these investigations is in connection with caseation in tuberculosis. The large amount of lipoids contained in the tubercle bacilli serves to inhibit the ferments liberated as a result of the disintegration of the cells, which explains the caseous lesions. It was found also that soaps prepared from the lipoids of the tubercle bacilli are markedly inhibitory to ferment action. The softening of the caseous masses brought about by iodine is explained by the fact that the unsaturated fatty acids which play an important role as antiferments become saturated with iodine and lose thereby their ferment inhibitory properties. It is probable that in gummata we deal with conditions similar to those found in caseous material of tuberculosis and the effect of iodine in syphilis may also be explained by its property to cause resolution of the gummata by removing the antiferments.

Doerr, of Vienna, closes a most excellent review of recent work on anaphylaxis or protein sensitization with the following paragraph:

"While it must be admitted that the action of those infective bacteria, which are not known to produce specific toxins, remains without explanation, and while the theories which have been developed by von Pirquet, Friedberger, Vaughan, Schittenhelm, Weicherdt, and others have opened up a new way to the understanding of incubation, fever, and crises, still it must be borne in mind that the premises of these theories do not possess the force of chemical facts. It has not been positively shown that the symptoms of anaphylaxis are due to the parenteral cleavage of proteins, that the true anaphylactic poison is



identical with that produced *in vitro*, and that both come from the antigen. Even if we agree with Dold, Sachs, and Ritz, that so far as the role of anaphylaxis in the infectious diseases is concerned, it is irrelevant from what matrix and by what processes the hypothetical anaphylactic poison is produced, even then all the difficulties are not removed. Numerous infecting agents are not anaphylactogens; they do not differ in their effects upon sensitized and non-sensitized animals; and even when there are differences, they are slight compared with those seen when the protein antigens derived from the higher plants are employed. The relatively simple structure of the bacterial proteins is the cause of this. Therefore it is questionable whether one has the right to explain the phenomena of the infectious diseases with serum sensitization as a starting-point. Moreover, the infections are not so monomorphic as some suppose from a superficial consideration."

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For a more complete discussion, the reader is referred to the original papers of Jobling, Peterson and Eggstein in Jour. Exp. Med., 1913-1916, also in the Johns Hopkins Hosp. Bull., 1915, xxvi, and Jour. Lab. Clin. Med., 1916, i, 172. Also Bronfenbrenner, Jour. Lab. Clin. Med., 1916, i, 573.

## PART II.

# PATHOGENIC MICROÖRGANISMS INDIVIDUALLY CONSIDERED.

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### CHAPTER XV.

#### THE PATHOGENIC MOLDS (HYPHOMYCETES, EUMYCETES) AND YEASTS (BLASTOMYCETES).

##### THE HYPHOMYCETES.

THE majority of the molds are not pathogenic for human beings, and interest us more as organisms which are apt to infect foodstuffs and media. Some are, however, true parasites, and produce a number of rather common diseases; for example, ringworm, favus, thrush, and pityriasis versicolor. Certain of the commoner molds (*mucor*, *aspergillus*) have also been reported from time to time as present in pathogenic conditions in man as well as in the lower animals. Many varieties have been found in plant diseases, and some may be a source of danger to man indirectly. Indeed, when they form poisonous substances, as in the infection of grain by *claviceps purpurea* (ergot poisoning), they are distinctly dangerous.

Paltauf reported the case of a man who died after enteritis with secondary peritonitis. The autopsy showed multiple abscesses in brain and lungs, besides the lesions in the intestines and peritoneum, in all of which a species of *mucor* was found. Two other cases of primary *mucor* infection in humans were reported by Furbringer. A number of species of *mucor* have been found in ear and eye infections; for example, *Mucor corymbifer* (Fig. 90) has been found in ophthalmia. A number of species of molds are pathogenic for lower animals. *Aspergillus* is found thus very frequently in lower animals, especially in birds, where a kind of pseudotuberculosis is often produced. Quite a number of similar cases have been reported in man, and it is supposed that the infection may be carried from birds to man. *Aspergillus nodulans* (branched sterigmata) and *aspergillus fumigatus* (Fig. 91) are the most frequent varieties found. *Penicillium minimum* (similar to *glaucum*, Fig. 89) has been found by Liebermann in inflammation of the ear.

The more common pathogenic forms for man are those producing the various hair and skin lesions mentioned above. These molds are

all classed with the fungi imperfecti. It has just been announced that "trench foot" is due to a mold. (See p. 27 for general characteristics of molds; see also Plate I.)

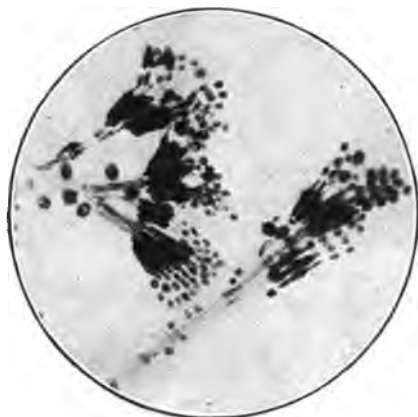


FIG. 89.—*Penicillium glaucum*. Gelatin culture. Spread stained with gentian violet. 500 : 1. (From Itzerott and Niemann.)

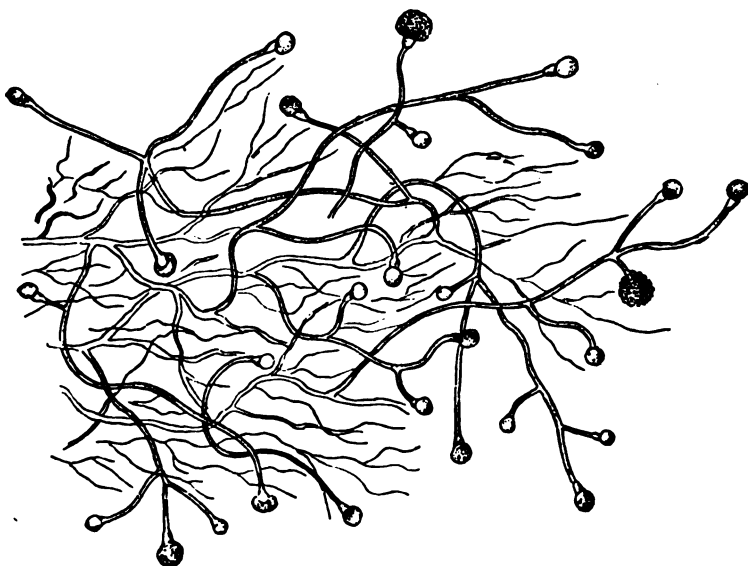


FIG. 90.—*Mucor corymbifer*, Coh. Mycelium with underlying branched carriers,  $2\frac{1}{2}\times$ . (After Lichtheim.)

**Trichophyton (Tinea, Ringworm Fungus).**—Ringworm of the body or hairless parts of the skin, *Tinea circinata*, and ringworm of the hairy parts, *Tinea tonsurans* (*Herpes tonsurans*) and *Tinea barbæ* or *Tinea sycosis*, are due to several species of the *imperfecti* group (p. 28).

**Methods of Examination.**—*Living Specimens.*—Place scales or hairs in warm liq. potassæ (20 per cent.) for a few seconds. Examine under cover-glass.

*Permanent Specimens.*—Remove fat with chloroform, then place material in formic acid and heat to boiling (two or three minutes). Remove acid by washing in distilled water, stain with Löffler's methylene blue. Wash, dehydrate in absolute alcohol, clear in xylol and mount in balsam.

Sabouraud's media are best for growth of the organism (p. 110). Upon this medium within half a dozen days septate mycelial threads with chlamydospores (p. 27) are seen. Reddish or brown pigment sometimes develops.

According to Sabouraud, whose conclusions are based on an extensive series of microscopic examinations of cases of tinea in man and animals, of cultivation in artificial media, and of inoculation on man and animals, there are two distinct types of the fungi causing ringworm in man—one with small spores ( $2\mu$  to  $3\mu$ ) which are known as *microspora*, and one with large spores ( $7\mu$  to  $8\mu$ ) which are called *megalospora*. They differ in their mode of growth on artificial media and in their pathological



FIG. 91.—*Aspergillus fumigatus*. Gelatin culture. Spread stained with gentian violet 500 : 1. (From Itzerott and Niemann.)

effects on the human skin and its appendages. The small spored type is the common fungus of *Tinea tonsurans* of children, especially of those cases which are rebellious to treatment, and its special seat of growth is in the substance of the hair. *T. megalosporon* (Fig. 92) is essentially the fungus of ringworm of the beard and of the smooth part of the skin; the prognosis as regards treatment is good. One-third of the cases of *T. tonsurans* of children are due to megalospora. The spores of *T. microsporon* are contained in a mycelium; but this is not visible, the spores appearing irregularly piled up like zoöglea masses; and, growing outside, they form a dense sheath around the hair. The spores of *T. megalosporon* are always contained in distinct mycelium filaments, which may either be resistant when the hair is broken up or fragile and easily breaking up into spores. The two types show distinct and constant characters when grown in artificial cultures. The cultures of *T. microsporon* show a downy surface and white color; those of *T. megalosporon* a powdery surface, with arborescent peripheral rays, and often a yellow-

ish color. Although the morphological appearances, mode of growth, and clinical effects of each type of trichophyton show certain characters in general, yet there are certain constant minor differences which point

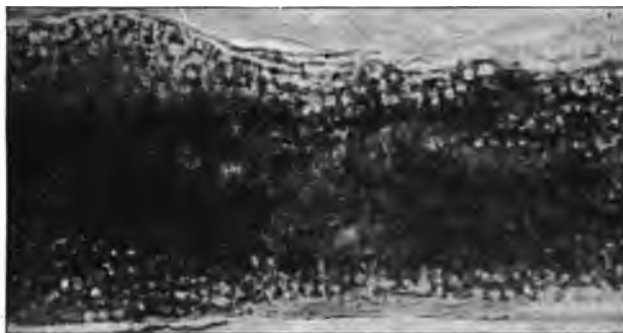


FIG. 92.—Hair riddled with ringworm fungus. *Megalosporon* variety.



FIG. 93.—These two half-plates show three months' growth on peptone-maltose agar of two megalosporon varieties of the ringworm fungus. Natural size.

to the fact that there are several different kinds of species of fungus included under each type. The species included under *T. microsporon* are few in number, and, with the exception of one which causes the common

contagious "herpes" of the horse, almost entirely human. The species of *T. megalosporon* are numerous and fall into several natural groups, the members of which resemble one another both from clinical and mycological aspects (Fig. 93). Cultures inoculated into guinea-pigs and other laboratory animals may produce infection.

**Achorion Schoenleinii (Favus).**—Favus is due to a fungus discovered by Schoenlein in 1839, and called by Remak *Achorion schoenleinii*. The disease is communicated by contagion, the fungus being often derived from animals, especially cats, mice, rabbits, and fowls; dogs also are subject to it. It grows much more slowly than the ringworm fungus, and is therefore not so quickly transmitted but it will surely infect careless, dirty people coming in daily contact with it. Want of cleanliness is a predisposing factor. The fungus seems to find a more favorable soil for its development on the skin of persons in weak health, more in those suffering from phthisis than from other diseases.

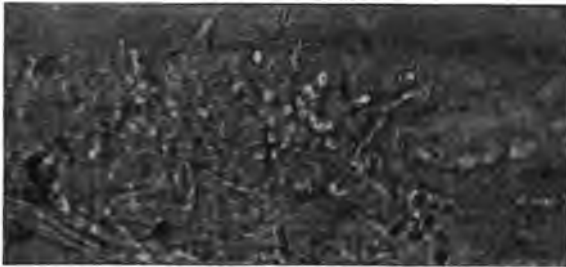


FIG. 94.—A portion of a favus-infected hair; magnified.

Favus produces tissue irritation of a very chronic course and of great resistance to treatment. The spores generally find their way into the hair follicles, where they grow in and about the hair (Fig. 94). It grows in the epidermis, the density of the growth causing pressure on the parts below, thus crushing out the vitality of the hair and giving rise to atrophic scarring. The disease shows a marked preference for the scalp, but no part of the skin is exempt, and even the mucous membranes are likely to be attacked. Kaposi has reported a case in which a patient suffering from universal favus died, with symptoms of severe gastrointestinal irritation, which was found after death to be due to the presence of the favus fungus in the stomach and intestines. Foster reported many cases of favus and ringworm of the nails in immigrants. On the scalp it first appears as a tiny sulphur-yellow disk or *scutulum*, depressed in the centre like a cup and pierced by a hair. This is the characteristic lesion. The cup shape is attributed by Unna to the fact that growth is more vigorous at the sides than at the centre. Under the microscope material from a scutulum teased out in a drop or two of 20 per cent. sodium hydrate solution (slightly heated) shows chiefly small doubly contoured, round or oval spores, single or in chains scattered throughout a dense network of fine threads among which on the edge of

the scutulum may be recognized distinct branched hyphæ with swollen ends.

The favus fungus is readily cultivated at the body temperature, less readily at room temperature, in the ordinary culture media, as agar, blood serum, gelatin, bouillon, milk, infusion of malt, eggs, potato. It shows a preference for slightly acid media (p. 110). The growth develops slowly and shows a preference to growth beneath the surface



FIG. 95.—Five-month-old colony of favus on peptone-maltose agar; actual size.

of the medium—except on potato, upon which it develops on the surface in layers. The characteristic form of growth is that of moss-like projections from a central body (Fig. 95). The color is at first grayish white, then yellowish. As seen under the microscope, ray-like filaments are developed, which divide into branches. The ends are often swollen or club-shaped, and there are various enlargements along the body of the filament. Several varieties of favus have been discovered.

Recently the Roentgen-ray treatment has been recommended for aborting the disease but this is not always practicable. Also specific vaccines have been tried but reports from their use are not yet satisfactory.

**Microsporon Furfur** (described by Eichstedt in 1846).—This organism, found in pityriasis versicolor, belongs to a group of fungi which, in contrast to the more parasitic fungi, favus and trichophyton, invades only the most superficial layers of the skin and does not give rise to any considerable pathological changes in the skin or hair. Although the vegetative elements of these fungi are much more numerous in the affected portions of the skin than is the case with the more parasitic species, they are not nearly as contagious as the latter.

By preference the organism attacks the chest, abdomen, back, and axillæ, less frequently neck and arms, while exceptionally it attacks also the face. The growth shows itself as scattered spots varying in color from that of cream-coffee to reddish-brown. The spots are readily scraped off and show fine lamellation or scaling. Occasionally the spots are confluent, and sometimes arranged in ring form like *Herpes tonsurans*.

In spite of their slight contagiousness this is one of the most frequent dermatomycoses. Although it is distributed widely over the earth, it is more frequently observed in southern than in northern countries.

Persons with a tender skin and a disposition to perspire freely are particularly affected by *Pityriasis versicolor*, and this is undoubtedly the only reason why the affection is so frequently observed in consumptives. Women are more frequently attacked than men, while children and old people are rarely affected.

The source of infection is unknown, since the absence of contagion has frequently been demonstrated. It seems likely that the spores of this fungus are so widely distributed that susceptible individuals are easily infected.

The arrangement of the fungus in the scales of epidermis is characteristic. The short and thick curved hyphæ ( $7\mu$  to  $13\mu$  long and  $3\mu$  to  $4\mu$  wide) surround large clumps of spores. The spores are coarse, doubly contoured ( $4\mu$  to  $7\mu$ ) and round. On staining with Ziehl's solution the spores are seen to contain deeply stained globules lying, in all probability, on the inner surface of the cell membrane. The rest of the protoplasm is but little stained, or not at all. One frequently finds that these globules are also found free; what their nature is does not appear; they are not found in cultures, the freshly developed spores showing only a single globular mass of protoplasm possessing a fine blue luster.

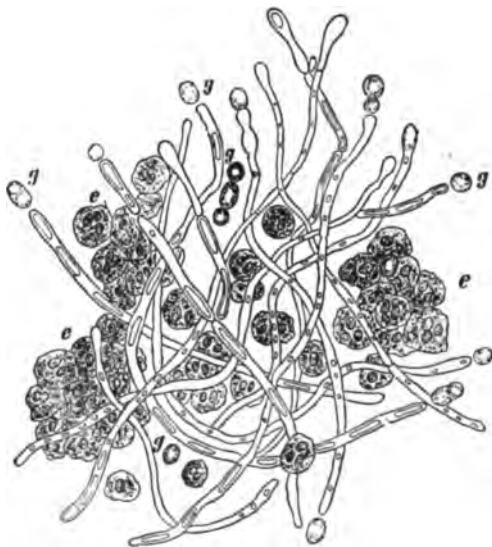


FIG. 96.—Inflammation of cornea by thrush (*Oidium albicans*), *g*, conidia; *e*, pus cells.  
(From Plaut, in Kollé and Wassermann.)

Cultures are obtained with difficulty and the growths cannot be transferred for many culture generations. The growth is said to be most characteristic on potato which shows yellowish-white colonies within four or five days.

**Thrush** (*Oöspora*, *Oidium Albicans*, *Monilia Albicans*) (Fig. 96).—Thrush, as is well known, occurs most frequently in the oral mucous membrane of infants during the early weeks of life. It is also found as a slight mycosis in the vagina, especially of pregnant women. In rare cases the disease attacks adults, and especially those whose system has been undermined by other diseases, such as diabetes, typhoid, etc. A few cases are recorded in literature in which this fungus has given rise to



constitutional disease. In these cases autopsy has shown abscesses in various parts of the body, such as the lungs, spleen, kidney and brain.

The tropical disease called sprue has been said by Baker to be due to *Monilia albicans*. Ashford thinks this disease is caused by a distinct species of monilia.

In the lesions of the disease as well as in cultures this fungus appears as both a yeast and a mycelium. It thus seems to stand between the true molds and the yeasts. The yeast cells are oval in form, about  $5\mu$  to  $6\mu$  long and  $4\mu$  wide, and can in no way be distinguished from other yeast cells either by their appearance or their method of propagation. The threads of the mycelium vary markedly in length and thickness, and show all intermediate forms between a typical and a budding mycelium. Cultures are easily obtained on artificial media, especially on those favorable for yeasts (p. 110).

Thrush is not much influenced by acids or alkalis. On the other hand, it is very susceptible to the common disinfectants, especially salicylic acid, corrosive sublimate, phenol, iodine, etc. This fact is made use of in local treatment.

**Sporotricha.**—Another of the molds classed with the *Fungi imperfecti* (family Mucedineæ) has been found to produce disease in man and some of the lower animals. The first variety described was by Schenk in 1898. It was declared one of the sporotricha by F. Smith. In 1900 Hektoen and Perkins described minutely a culture isolated from another case and gave the organism the name *Sporotrix* (Sporotrichon) *schenki*.

Since the disease was made more thoroughly known by the studies of Beurmann and Gougerot and others, many cases have been reported in various parts of the world, and several new species of sporotricha have been described. Beurmann and Gougerot first described the species so far most frequently reported, that is, *Sporotrichon beurmanni*, which is so similar to *Sporotrichon schenki* that they are classed now by most observers as one species. So several of the other species described may prove on further study to be but one species.

Earlier the disease was often mistaken for tuberculosis or syphilis because of the similarity of the lesions which in general are slowly growing granulomata ending in degeneration. When it was found that the disease responds readily to treatment with potassium iodide and other iodine combinations, but not to mercury salts, it became comparatively easy to corroborate the diagnosis. In 1912 Rudiger published a review of the cases reported to date. In the latter part of 1915 Meyer reported that the disease is very common in certain parts of the United States, in domesticated animals, especially in horses, but that human beings do not readily contract the disease from them.

**Morphology.**—In the pus from the lesions only oval, highly refractile conidia or spores ( $3\mu$  to  $4\mu \times 1.6\mu$  to  $3\mu$ ) are seen and these generally sparingly. They may lie within the tissue cells. They are Gram-positive. In cultures these spores grow out into a branching irregularly septate mycelium (the hyphæ about  $1\mu$  thick), and the new conidia

are formed simply at the sides and ends of the hyphæ without definite fruiting organs. The conidia often occur in whorls. (Plate I, Fig. 5.)

**Cultivation.**—They grow readily on ordinary media, but better when it is slightly acid. Sabouraud's medium (p. 110) is most favorable. They grow both at room and at incubator (36° C.) temperature. At 20–25° C. growth is seen in about four days, and at 36° C. in about forty-eight hours. Minute, fluffy, snowflake-like colonies appear which slowly become brown or brown-black, and, when in a mass, convoluted. Gougerot found that by letting a drop of the pus flow over a glass slide and keeping in a moist atmosphere, the sprouting of the spores may be followed under the microscope in two or three days. Agglutination and complement-fixation have been studied by Widal and Abrami (1908). Their results, which are given in the next paragraphs, await further corroboration.

**Agglutination.**—Homogeneous emulsions of the spores are made from cultures on Sabouraud's medium six to twelve weeks old. A large amount of the cultures is removed and rubbed until dry in a mortar. To this, drop by drop, is added several cubic centimeters of normal salt solution, while continuing to rub. The suspension is filtered through moistened filter paper. The filtrate contains only free spores which in positive cases agglutinate in from fifteen to sixty minutes in dilutions of 1 to 150 to 1 to 1800, generally 1 to 300 to 1 to 400. Agglutination with the sera of other mold and yeast infections may occur to less extent.

**Complement-fixation** is said to be not specific for the different mold and yeast infections.

**Pathogenesis.**—Cultures or pus injected subcutaneously or intraperitoneally into lower animals such as mice, rats, and dogs produce granulomatous lesions similar to those found in man.

In man the lesions vary from superficial, non-ulcerative gummæ to deep visceral abscesses. Sometimes the diagnosis is difficult to determine. According to Gougerot the following points help in differentiation:

1. Many lesions with general good health.
2. Lesions begin as indolent swellings which gradually become large gummæ, on which one or several small ulcers with violet edges may appear. These ulcers discharge a thin, yellowish, shiny pus, and the centre gradually becomes cicatrized with a persistence of the abscess under the skin. Generally there is no adenitis.
3. Microscopic and cultural examinations reveal the organisms. Large quantities of the pus should be inoculated into each culture tube which should not be closed with rubber cap. The dry method should also be tried. Direct smears are not satisfactory.
4. Serum diagnosis may be tried.
5. Animal inoculations give positive results.
6. Treatment with potassium iodide. This produces a cure unless given very late in the disease or unless the mucous membranes are extensively affected. Some cases need treatment for two or three months before improvement is seen. Occasionally death occurs.

## PATHOGENIC YEASTS.

The pathogenic blastomycetes (see p. 28 for general characteristics of yeasts; see also Plate I, Figs. 6 and 7) may be briefly summarized as follows:

*Saccharomyces busse* is the name given to a yeast isolated in 1894 by O. Busse from an abscess in the tibia of a thirty-one-year-old woman who died thirteen months after the first symptoms appeared. The autopsy showed numerous broken-down nodules on several of the bones, and in the lungs, spleen, and kidneys. The yeast was cultivated from all these foci.

*Saccharomyces tumefaciens* was isolated in 1895 by Curtis. The patient was a young man showing multiple tumors on the lips and neck having the gross appearance of softened myxosarcomata.

This yeast is pathogenic for rats, mice, and dogs, only slightly so for rabbits, and not at all for guinea-pigs.

Various similar cases have since been described, a number of them becoming generalized, and ending fatally. In generalized blastomycosis the lung seems frequently to be the seat of primary infection.

The cases described first by Rixford and Gilchrist as coccidiosis due to "*Coccidioides immitis*"—thought to be a protozoön—may be classed here, since Ophüls, Oliver, and others have shown that the "coccidium" forms hyphæ and elliptical forms on culture. Cummins and Sander have shown that cultures from fresh tissue grow slowly (appearing in from 7 to 12 days in primary cultures) but with ease on the usual laboratory media. The growth is more mold-like than yeast-like, except on potato, when budding yeast-like cells are produced. Dogs, rabbits, and guinea-pigs are susceptible to the fungus and show lesions similar to those in human beings.

Zinsser isolated an organism similar to those described by Ophüls and Oliver from an abscess of the back. Brown and Cummins state, after an extensive study of "coccidioid" disease, that the species of yeast causing this group of cases is distinct from that caused by the more definitely budding type of yeast.

A typical case of systemic blastomycosis, reported by Fontaine, Hasse, and Mitchell is accompanied by very good illustrations of tissue sections. Fig. 97 shows the characteristic microscopic appearance of the lung lesion.

Several cases of ophthalmia have been described as due to yeasts. Recently Jackson and others have added to the reports of cases of eye infection by yeasts. Even "trachoma" has been said to be caused by this organism.

Buschke isolated a yeast from a cervical discharge in which no gonococci were present. The yeast was pathogenic for guinea-pigs.

In 1895 Tokishige reported an epidemic quite common among horses in Japan, known as "Japanese worm," "benign worm," or "pseudo-worm," which is caused by one of the saccharomyces. This disease begins in the skin in the form of hard, painless nodules from the size of a pea to that of a walnut. These break down and give rise to gradually

extending ulcers. Pure cultures of the saccharomyces are pathogenic chiefly for horses, not for rabbits, guinea-pigs, or hogs. In the districts where the disease prevails among horses it is also frequently seen in cattle.

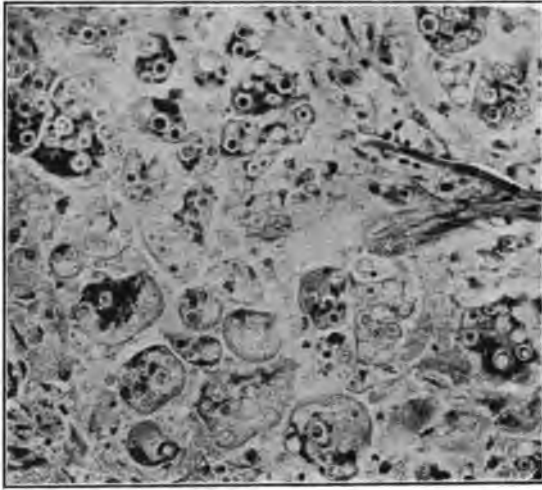


FIG. 97.—Section of lung.  $\times 150$ ; blastomycetes in large syncytial cell masses. (From Fontaine, Hasse, and Mitchell.)

Shortly after Tokishige's publication a similar disease occurring in horses in Italy and southern France was identified as being caused by saccharomyces. Cultures of this yeast, however, differ somewhat



FIG. 98.—Blastomycosis in infant aged eight months, showing lesion on left cheek. (Kessler.)

from that obtained in Japan, so that Busse is inclined to regard the two as two different species of blastomycetes.

Kartulis, in Alexandria, described about a hundred cases of a skin

affection occurring in the gluteal regions of men and characterized by an elongated finger-like swelling, which breaks and emits a purulent discharge, forming an unhealed sinus. In the discharge and surrounding tissues are numerous blastomycetes which Kartulis after cultivation and study considered a variety of the ordinary fermenting yeast (*Saccharomyces cerevisiæ* Hansen). The cases were cured by excising the growth.

Kessler reported a skin lesion in an infant (Fig. 98) probably due to a similar blastomycete, since the lesions healed after treatment with potassium iodide. The description of the yeast isolated is too incomplete to identify it.

Some years ago the attempt was made to connect the development of cancerous growth with blastomycetes. This was due in a measure to a certain similarity between the yeasts and the cell inclusions or so-called "parasites" of cancer, and further, to the fact that when yeasts are injected into the animal body tumor-like nodules are often developed at the site of inoculation and in the internal organs. But these nodules are not tumors in the pathological sense of the term, but merely masses of blastomycetes mixed to a very variable degree with inflammatory tissue proliferations.

Yeasts are Gram-positive, or Gram-amphophile. They stain readily with other stains in the young state. Older forms stain very irregularly. They are cultivated—sometimes with difficulty directly from the tissues—on ordinary media, but best on media made slightly acid (p. 110). Some pathogenic varieties grow best at blood heat. Most varieties grow easily at room temperature.

The important part played by yeasts in certain industries is treated in Part III.

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## CHAPTER XVI.

### THE PYOGENIC COCCI.

#### THE STAPHYLOCOCCI (MICROCOCCI).

PRACTICALLY all microorganisms have been shown by experiment to induce, under certain conditions, the formation of pus by their products when inoculated into the animal body; but, while this has been demonstrated, the researches of bacteriologists show that only a few species are usually concerned in the production of acute abscesses in man. Of these the two most important, by reason of their frequent occurrence and pathogenic power, are *Staphylococcus aureus* and *Streptococcus pyogenes*. These two organisms are often found in the same abscess; thus, Passet, in 33 cases of acute abscess, found *Staphylococcus aureus* alone in 6, *aureus* and *albus* associated in 11, *albus* alone in 4, *albus* and *citreus* in 2, *Streptococcus pyogenes* alone in 8, *albus* and *Streptococcus* in 1, and *albus*, *citreus*, and *Streptococcus* in 1. The staphylococcus is likely to enter as a mixed infection into most infections due to other bacteria, and is almost always met with in all inflammations of the skin and mucous membranes or in cavities connected with them.

Staphylococci were first obtained from pus by Pasteur in 1880. In 1881 Ogston showed that they frequently occurred in abscesses, and in 1884 Rosenbach fully demonstrated their etiological importance in circumscribed abscesses, osteomyelitis, etc. Of the staphylococci those producing yellow and white pigments are by far the most important since they are the pathogenic varieties.

**The *Staphylococcus (Pyogenes) Aureus*.**—The *Staphylococcus aureus* is one of the commonest pathogenic bacteria, being usually present in the skin and mucous membranes, and is the organism most frequently concerned in the production of acute, circumscribed, suppurative inflammations.

**Morphology.**—Small, spherical cells, having an average diameter of  $0.7\mu$  to  $0.9\mu$ , occurring solitary, in pairs as diplococci, in short rows of three or four elements, or in groups of four, but most commonly in irregular masses, simulating clusters of grapes; hence the name *staphylococcus*. (See Fig. 99.)

**Staining.**—It stains quickly in aqueous solutions of the basic aniline colors and with many other dyes. When previously stained with aniline gentian violet it is not decolorized by Gram's method. When slightly stained each sphere frequently is seen to be already dividing into two semispherical bodies.

**Biology.**—The *Staphylococcus (pyogenes) aureus* is a non-motile, aërobic, facultative anaërobic micrococcus, growing at a temperature

from 8° to 43° C., but best at 25° to 35° C. The staphylococci grow readily on all the common laboratory media, such as milk, bouillon, nutrient gelatin, or agar. A slightly alkaline reaction to litmus is best for the growth of the staphylococci, but they also grow in slightly acid media.

**Cultivation.—Growth in Nutrient Bouillon.**—The growth of the staphylococcus is rapid, reaching about 500,000,000 per c.c. at the end of twenty-four hours at 30° C. The bouillon is cloudy and frequently has a thin pellicle. Later a slimy sediment forms. The odor is disagreeable. In peptone-water, growth occurs with indol production.

**Growth on Gelatin.**—Grown on gelatin plates it develops, at room temperature, within forty-eight hours, punctiform colonies, which when examined under a low-power lens, appear as circular disks of a pale brown color, somewhat darker in the centre, and surrounded by a smooth border. The colonies grow rapidly. The appearance of the growth is most characteristic. Immediately surrounding the colonies,

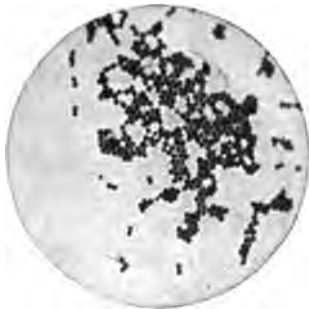


FIG. 99.—*Staphylococcus*.  
× 1100 diameters.

which are of a pale golden yellow color, there is a pitting of the surface of the gelatin, due to its liquefaction. By suitable light a number of these shallow depressions with sharply defined outlines may be seen on the gelatin plate, having a diameter of from 5 to 10 mm., in the centres of which lie the yellow colonies. Later the liquefaction becomes general, the colonies running together. In stab cultures in gelatin a white confluent growth at first appears along the line of puncture, followed by a funnel-shaped liquefaction of the medium, which rapidly

extends to the sides of the test-tube. At the end of two days the yellow pigmentation begins to form, and this increases in intensity for eight days. Finally, the gelatin is completely liquefied, and the staphylococci form a golden-yellow or orange-colored deposit at the bottom of the tube. Under unfavorable conditions the *Staphylococcus aureus* gradually loses its ability to make pigment and to liquefy gelatin. The liquefaction is due to a ferment called gelatinase formed by the staphylococci. It may be separated from the cocci by filtration (Loeb).

**Growth on Agar.**—In streak and stab cultures on agar a whitish growth is at first produced, and this at the end of a few days becomes a faint to a rich golden yellow on the surface. The yellow pigmentation is produced only in the presence of oxygen; colonies formed at the bottom of a stab culture or under a layer of oil remain white.

**Milk.**—Milk is coagulated at the end of from one to eight days.

**Potato.**—The staphylococci grow readily on potato and produce abundant deep colored pigment.

**Growth on Löffler's Solidified Blood Serum.**—Growth vigorous, with fairly good pigment production. Some varieties slowly liquefy the serum.

**Growth on Blood Agar.**—If nutrient agar to which a little animal blood has been added is streaked with staphylococci there appears, at the end of twenty-four hours at 35° C., about the growth a clear zone, owing to the hemolytic effect of the staphylococcus products.

**Acids Produced.**—In media containing carbohydrates there is, as a result of the growth of the *Staphylococcus aureus*, a *production of acid* in considerable quantities, consisting chiefly of lactic, butyric, and valerianic acids. These acids have been supposed to play a part in the production of pus, in which, according to some observers, they are often present. No gas is formed.

**Resistance.**—The staphylococcus is distinguished from most other non-spore-bearing pathogenic bacteria by its greater power of resistance to outside influences, desiccation, etc., as well as to chemical disinfectants. Cultures of the *Staphylococcus pyogenes* in gelatin or agar retain their vitality for a year or more. Suspended in water its thermal death-point varies with different cultures and averages about two hours at 50° C., one-half hour at 60° C., ten minutes at 70° C., and five minutes at 80° C. Upon silk threads and in media rich in organic matter its resistance is greater, but subjected to 80° C., for thirty minutes or boiling for two minutes it is almost surely killed. Cold has but little effect. Thirty per cent. of the organisms remained alive after being subjected by us to freezing in liquid air for thirty minutes. These are average figures. Some cultures are more resistant than others.

They are quite resistant to direct sunlight and drying. Dried pus contains living staphylococci for weeks and even months, and they can be found alive in the fine dust of the air in living rooms and operating rooms.

Resistance to chemicals is given in Part III.

**Pigment Formation.**—Pigment formation, already described, is considered within a limited species characteristic. Thus different strains of *Staphylococcus aureus* produce a pigment varying from a pale brown to a deep golden yellow. It usually becomes less intense upon prolonged cultivation. The pigment is classed as a lipochrome (Schneider). It is soluble in alcohol, chloroform and ether.

In order to test the amount of color produced, Winslow and Rogers recommend the following method: A portion of the growth is removed on a loop needle and spread out on white drawing paper with a rough surface. After drying at room temperature the color is compared with a standard color chart.

**Pathogenesis.**—The pathogenic effect of the *Staphylococcus (pyogenes) aureus* on test animals varies considerably, according to the mode of application, the virulence of the special culture employed and the species of animal used. In man a simple rubbing of the surface of the unbroken skin with pus from an acute abscess is, as a rule, sufficient to produce a purulent inflammation, and the introduction of a few germs from a septic case into a wound may lead to a fatal pyemia. These conditions can only be reproduced in lower animals with difficulty, and by the inoculation of large quantities of the culture. Small subcutaneous injections, or the inoculation of open wounds in mice, guinea-pigs, and



rabbits, are commonly without result; occasionally abscess formation may follow at the point of inoculation, which usually ends in recovery. The pus-producing property of the organism is exhibited in proportion to the virulence of the culture employed. Slightly virulent cultures, which constitute the majority of those obtained from pus taken from the human subject, when injected subcutaneously in large quantities (several cubic centimeters of a fresh bouillon culture) into rabbits or guinea-pigs, give rise to local pathological lesions—acute abscesses. When virulent cultures are used—usually those recently isolated from human infections—0.5 c.c. of a fresh bouillon culture is sufficient to produce similar results. The abscesses generally heal without treatment; sometimes the animals die from marasmus in consequence of the suppurative process. In intraperitoneal inoculations the degree of virulence of the culture employed is still more evident in the effects produced. The animals usually die in from two to nine days. The most characteristic pathological lesions are found in the kidneys, which contain numerous small collections of pus, and under the microscope present the appearances resulting from embolic nephritis. Punctiform, whitish-yellow masses of the size of a pea are found permeating the pyramids. Many of the capillaries and some of the smaller arteries of the cortex are plugged up with thrombi, consisting of micrococci. Metastatic abscesses may also be observed in the joints and muscles. The micrococci may be recovered in pure cultures from the blood and the various organs; but they are not numerous in the blood and are often difficult to demonstrate microscopically. Intravenous inoculations of animals are followed by similar pathological changes. Orth and Wyssokowitsch first pointed out that injection of staphylococci into the circulation of rabbits, whose cardiac valves have previously been injured, produced ulcerative endocarditis. Subsequently Weichselbaum, Prudden, and Fränkel and Sängner obtained confirmatory results, thus establishing the fact that when the valves are first injured, mechanically or chemically, the injection into a vein of a pure culture of *Staphylococcus aureus* gives rise to a genuine ulcerative endocarditis. It has been further shown by Ribbert that the same result may be obtained without previous injury to the valves by injecting into a vein the staphylococcus from a potato culture suspended in water. In his experiments not only the micrococci from the surface, but the superficial layer of the potato were scraped off with a sterilized knife and mixed with distilled water and the successful result is ascribed to the fact that the little agglomerations of micrococci and infected fragments of potato attach themselves to the margins of the valves more readily than isolated cocci would do. Not infrequently, also, in intravenous inoculations of young animals there occurs a localization of the injected material in the marrow of the small bones. This may take place in full-grown animals when the bones have been injured or fractured. The experimental osteomyelitis thus produced has been demonstrated to be anatomically analogous to this disease in man. An increase in virulence of certain strains may be obtained by successive passage through susceptible animals.

**Toxic Substances Produced.**—Filtrates of cultures contain toxic substances. Injected into the peritoneal cavity they excite peritonitis. Under the skin they produce infiltration or abscess formation. In the blood they injure both the red and white corpuscles.

Cultures of the staphylococcus, when sterilized by boiling and injected subcutaneously, produce marked positive chemotaxis and often local abscesses. Leber found also that sterilized cultures introduced into the anterior chamber of the rabbit's eye would bring about a fibropurulent inflammation, the cornea becoming infiltrated, and perforation alongside of the sclerotic ring finally taking place. This was followed by the formation of pus in the anterior chamber and recovery. These local changes follow the inoculation of small quantities only of the dead cultures; but when large amounts are injected into a vein or into the abdominal cavity, toxic effects are produced. The hemolytic effects of certain products of virulent staphylococci have recently been studied. In cultures they can be detected about the third or fourth day of incubation and reach their maximum on the ninth to fourteenth day. Virulent staphylococci are more apt to produce this substance than the non-virulent, but there is no definite rule.

The specific hemolysin, known as staphylolysin, is destroyed by heating for twenty minutes at 56° C. An antibody for this is formed by inoculating animals with culture filtrates. A substance called leukocidin is produced which injures leukocytes, it also produces an antibody. The gelatin-liquefying enzyme — gelatinase — has already been mentioned.

**Occurrence in Man.**—The staphylococcus (*Staphylococcus aureus*) has been demonstrated not only in furuncles and carbuncles, but also in various pustular affections of the skin and mucous membranes—impetigo, sycosis, purulent conjunctivitis and inflammation of the lacrimal sac; in acute abscesses formed in the lymphatic glands, the parotid gland, the tonsils, the mammæ, etc.; in metastatic abscesses and purulent collections in the joints; in empyema, infectious osteomyelitis, ulcerative endocarditis, pyelonephritis, abscess of the liver, phlebitis, meningitis, etc. It is one of the chief etiological factors in the production of pyemia in the various pathological forms of that condition of disease. It is remarkable how many staphylococci may be present in the blood without a fatal result, if the source of infection is removed. We met with one case in which over 800 staphylococci were present in 1 c.c. of blood. A week later only 5 were found. The patient finally died from pneumonia.

Not all persons are equally susceptible to infection by the staphylococcus; those who are in a cachectic condition or suffering from constitutional diseases, like diabetes, are especially predisposed to infection. In healthy individuals certain parts of the body, as the back of the neck and the buttocks, are more liable to be attacked than others, with the production of furuncles, carbuncles, etc. In persons in whom sores are readily caused, in consequence of disturbances of nutrition, as in exhausting diseases, the micrococci settle at the points of least resistance.

Such conditions are present in the bones of debilitated young children, in fractures, and in injuries in general.

**Immunity.**—Rabbits have been rendered immune by means of inoculations with either dead or living cultures. Unless the inoculations are carefully made the animals frequently succumb. The staphylococci injected into an immunized animal are more rapidly taken up by the leukocytes than when injected into an untreated animal. (See Opsonins, p. 218.)

A serum having some protective power has also been elaborated.

Hiss claims good results from the use of leukocyte extracts in animals infected with *Staphylococcus aureus*. (See p. 225.)

**Therapeutic Use of Vaccine.**—The treatment of abscesses, boils, and other localized staphylococcus infections as well as general infections by injections of repeated doses of vaccine is considered in Part III.

**Staphylococcus (Pyogenes) Albus.**—It is morphologically identical with the *Staphylococcus (pyogenes) aureus*, and is probably the same organism which has lost the property of producing pigment. On the average it is somewhat less pathogenic and seldom produces pyemia or grave infections. The surface cultures upon nutrient agar and potato have a milk-white color. Its biological characters are not to be distinguished from the *Staphylococcus aureus*.

The majority of bacteriologists agree with Rosenbach, that the aureus is found at least twice as frequently in human pathological processes as the albus.

**Staphylococcus Epidermidis (Albus) (Welch).**—Probably identical with the *Staphylococcus (pyogenes) albus*. With reference to this micrococcus, Welch says: "So far as our observations extend—and already they amount to a large number—this coccus may be regarded as nearly, if not quite, a constant inhabitant of the epidermis. It is now clear why I have proposed to call it the *Staphylococcus epidermidis albus*. It possesses such feeble pyogenic capacity, as is shown by its behavior in wounds as well as by experiments on rabbits, that the designation *Staphylococcus pyogenes albus* does not seem appropriate. Still I am not inclined to insist too much upon this point, as very probably this coccus—which has hitherto been unquestionably identified by others with ordinary *Staphylococcus pyogenes albus* of Rosenbach—is an attenuated or modified form of the latter organism, although, as already mentioned, it presents some points of difference from the classical description of the white pyogenic coccus."

According to Welch, this coccus differs from the *Staphylococcus albus* in the fact that it liquefies gelatin more slowly, does not so quickly cause coagulation in milk, and is far less virulent when injected into the circulation of rabbits. It has been shown by the experiments of Bossowski and of Welch that this microorganism is very frequently present in clean wounds, and that usually it does not materially interfere with the healing process, although sometimes it appears to cause suppuration along the drainage tube, and is the common cause of "stitch abscesses."

**Staphylococcus (Pyogenes) Citreus.**—Isolated by Passet (1885) from the pus of acute abscesses, in which it is occasionally found in association with other pyogenic cocci. It is distinguished from the other species only by the formation of a lemon-yellow pigment.

**Other Staphylococci.**—Other varieties have been occasionally met with which differ in some respects from the typical varieties. This difference may be in the fact that they liquefy gelatin more slowly or not at all, or in pigment formation, or in agglutination, or in still other respects. None of these varieties seem to be of importance.

### THE MICROCOCCUS TETRAGENUS.

This organism was discovered by Gaffky (1881). It is not infrequently present in the saliva of healthy individuals and in the sputum of consumptive patients. In sputum it is sometimes an evidence of mouth contamination rather than lung infection. It has been observed repeatedly in the walls of cavities in pulmonary tuberculosis associated with other pathogenic bacteria, which, though playing no part in the etiology of the original disease, contribute, doubtless, to the progressive destruction of the lung. Its pyogenic character is shown by



FIG. 100.—*Micrococcus tetragenus*. Stained with methylene blue.  $\times 1000$  diameters.

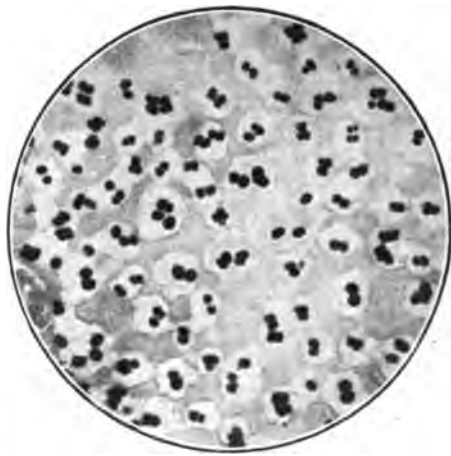


FIG. 101.—*Micrococcus tetragenus* from peritoneal fluid. Stained with fuchsin. (Fränkel.)  $\times 1000$  diameters.

its occasional occurrence in the pus of acute abscesses. Its presence has also been noted in the pus of empyema following pneumonia.

**Morphology.**—Micrococci having a diameter of about  $1\mu$ , which divide in two planes, forming tetrads, and bound together by a transparent, gelatinous substance, enclosing the cell like a capsule. In cultures the cocci are seen in various stages of division as large, round cells in pairs of oval elements, and in groups of three and four (Figs. 100 and 101). When the division is complete they remind one of sarcinae in appearance, except that they do not divide in three directions and are not built up like diminutive cotton bales (see also Plate II, Fig. 4, and Plate III, Fig. 11).

**Staining.**—This micrococcus *stains* readily with the ordinary aniline dyes; the transparent gelatinous envelope is only feebly stained. It is not decolorized by Gram's method.

**Biology.**—The growth of this micrococcus is slow under all conditions. It grows both in the presence and absence of oxygen; it grows best from 35° to 38° C., but may be cultivated also at the ordinary room temperature—about 20° C.

**Growth on Gelatin.**—On gelatin plates small, white colonies are developed in from twenty-four to forty-eight hours, which, when examined under a low-power lens, are seen to be spherical or lemon-shaped, grayish-yellow disks, with a finely granular or mulberry-like surface, and a uniform but somewhat roughly dentated border. When the deep colonies push forward to the surface of the gelatin they form white, elevated, drop-like masses, having a diameter of 1 to 2 mm. In gelatin stick cultures the gelatin is not liquefied.

**Growth on Agar and Blood Serum.**—The colonies appear as small transparent, round points, which have a grayish-yellow color and are slightly elevated above the surface of the medium.

**Pathogenesis.**—Subcutaneous injections of a culture of this micrococcus in minute quantity is usually fatal to white mice. The micrococci are found in comparatively small numbers in the blood of the vessels and heart, but are more numerous in the spleen, lungs, liver, and kidneys. Intraperitoneal injections given to guinea-pigs and mice are followed by purulent peritonitis, beautifully formed cocci in groups of four being obtained in immense numbers from the exudate. Rabbits and dogs are not affected by large doses of a culture subcutaneously or intravenously administered.

In man it is generally non-pathogenic, except in the conditions already cited. It has been obtained by us as the only organism in a case of chronic conjunctivitis.

The serum from immunized cases has not been used therapeutically in human infection. Vaccines may be employed as with staphylococci.

### THE STREPTOCOCCI.

The streptococci in their relation to human infection outweigh in importance most other disease-producing organisms. Under this name must be included not only the streptococci which excite inflammation in man, but all non-motile, non-spore-bearing spherical bacteria which divide, as a rule, in one plane only and remain attached in longer or shorter chains. Owing to the variations in morphology, cultural characteristics and virulence of this group of bacteria it has so far been impossible to give absolutely satisfactory differentiation of varieties in the group. The relation between pathogenicity and other characteristics is not clear-cut. The relation of the pneumococcus to the group has not been decided. The recent work of Rosenow on the transmutation of streptococci and pneumococci calls for confirmation. Such a transformation has not been proved to occur in any other types

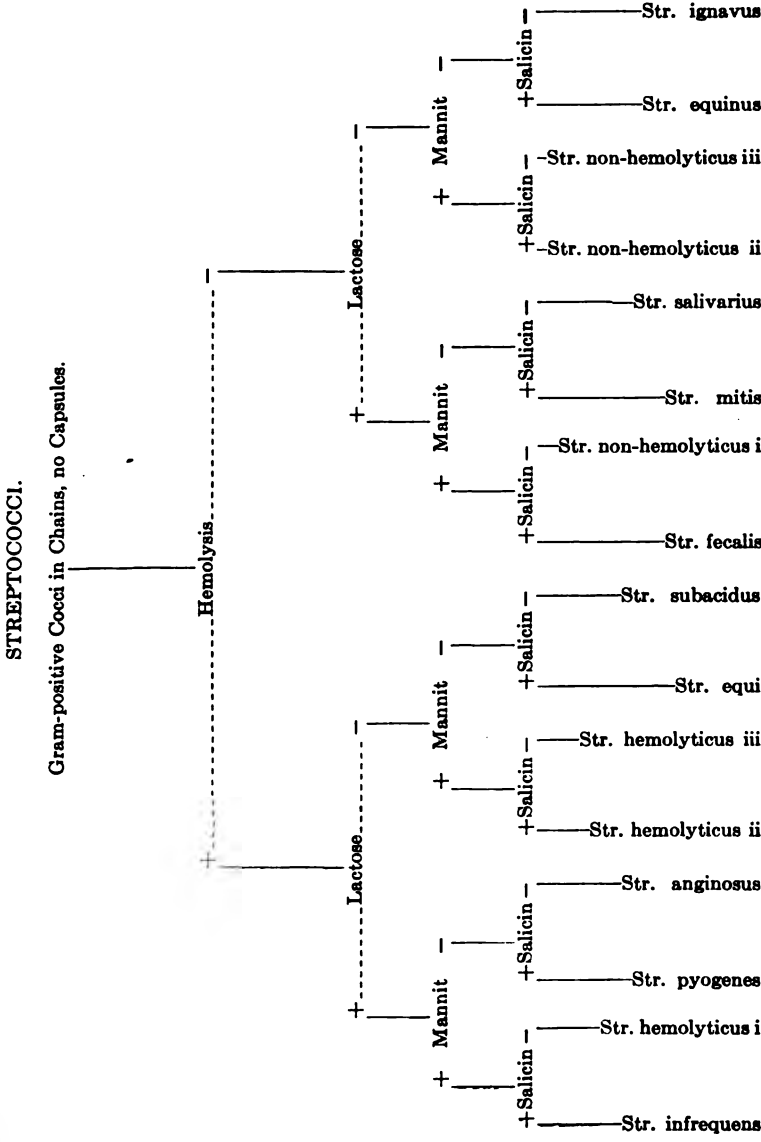
as distinct as these have appeared to be. The first classification was based upon pathogenicity and morphology. The first pathogenic streptococci were discovered by Koch in stained sections of tissue attacked by septic processes, and by Ogston in the pus of acute abscesses (1881). Pure cultures were obtained by Fehleisen (1883) from a case of erysipelas. The cultural and pathological characters were studied by him and it was shown to be capable of producing erysipelas in man. Rosenbach (1884) and Krause and Passet (1885) isolated the streptococcus from the pus of acute abscesses and gave it the name of *Streptococcus pyogenes*. It was first thought that the streptococci of erysipelas, of acute abscesses, of septicemia, of puerperal fever, etc., belonged each to a different species, because they seemed to possess differences in their biological and pathological characteristics, according to the source from which they were obtained. But it is now thought that the slight differences among the majority of streptococci from these diseases are but acquired variations of organisms derived from the same species.

The first grouping of the pathogenic forms on biochemical characteristics was made by Schottmüller in 1903. His three types were: (1) *Streptococcus pyogenes* or *erysipelatus*, which shows hemolysis on blood agar plates; (2) *Streptococcus mitior* or *viridans*, which produces a green halo about the colonies on blood agar plates, and (3) *Streptococcus mucosus* which produces a mucoid growth and a dark green zone. Park and Williams (1905) and others since then, showed that *Streptococcus mucosus* should be placed with the pneumococci under the common name pneumococcus mucosus. Gordon (1903) and Andrews and Horder (1906), using nine test substances on over a thousand strains, divided streptococci from various sources into nine groups. This work has been for the most part corroborated, but there has been much unfavorable criticism of such a classification based only on biochemical reactions. Serological reactions while encouraging, have not yet given clear-cut results. They have special importance in helping to indicate the proper serum to use in infections. Lyall recently has repeated the work with the carbohydrates and has tried to show the relationship between the characteristic carbohydrate reaction and the reactions on erythrocytes, but evidence being gathered by Krumwiede and others tends to show that the production of methemoglobin is not clear cut enough to be used as a primary basis for classification. Holman's classification (page 254) may be given as a later type of classification based on erythrocyte and carbohydrate reactions.

Dextrose and saccharose are not used in differential tests, since practically all streptococci ferment these sugars.

**Streptococcus Pyogenes.**—Under this heading are included all those streptococci that produce hemolysis and ferment dextrose, lactose, saccharose, and salicin but that do not ferment mannit.

**Morphology.**—The cocci, when fully developed are spherical or oval. They have no flagella or spores. They vary from  $0.4\mu$  to  $1\mu$  in diameter. They vary in dimensions in different cultures and even in different parts



of a single colony. They multiply by binary division in one direction only, forming chains of eight, ten, twenty, and more elements, being, however, often associated distinctly in pairs. On solid media the cocci occur frequently as diplococci, but usually they grow in longer or shorter chains. Frequently certain cocci exceed their fellows greatly in size,



FIG. 102.—Streptococci in peritoneal fluid, partly enclosed in leukocytes.  $\times$  1000 diameters.



FIG. 103.—Streptococcus growing in long chains in bouillon culture.  $\times$  1000 diameters.

especially in old cultures, when they may be considered the result of involution processes. Hueppe formerly called these arthrospheres. Some varieties have distinct capsules when growing in the blood and in blood-serum media.

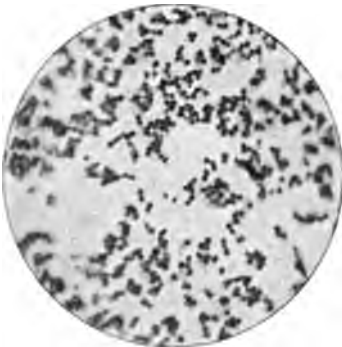


FIG. 104.—Streptococci from solidified serum culture appearing mostly in diplococci.  $\times$  1000 diameters.



FIG. 105.—Streptococci in throat exudate smeared on cover-glass.  $\times$  1000 diameters.

**Staining.**—They *stain* readily by aniline colors and the pyogenic varieties give a positive reaction by Gram's method. Some species, mostly saprophytic, growing in short chains are negative to Gram's stain.

**Biology.**—Streptococci of this type grow readily in various liquid and solid culture media. The most favorable temperature for their development is from  $30^{\circ}$  to  $37^{\circ}$  C., but they multiply rather freely at ordinary



room temperature—18° to 20° C. They are facultative anaërobes, growing both in the presence and absence of oxygen.

**Cultivation.—Growth on Gelatin.**—Tubes of gelatin which have been inoculated with these streptococci by puncture with a platinum needle show on the surface no growth beyond the point of entrance. In the depth of the gelatin on the second or third day a distinct, tiny band appears with granular edges or made up of granules. These granules may be very fine or fairly coarse. They are nearly translucent, with a whitish, yellowish, or brownish tinge. With characteristic cultures the gelatin is *not liquefied*.

**Growth on Agar.**—On agar plates the colonies are visible after twelve to thirty hours' growth at 37° C., and present a beautiful appearance when magnified sufficiently to see the individual cocci in the chain. The colonies are small, not averaging over 0.5 mm. in diameter (pin-head). From different sources they vary in size, thickness, mottling, color, and in the appearance of their borders. The streptococcus growing in short chains in bouillon shows but little tendency to form true loops, but rather projecting rows at the edges of the colonies, while those growing in long chains show beautiful loops, which are characteristic of this organism.

**Growth in Bouillon.**—Most streptococci of this type grow well in slightly alkaline bouillon at 37° C., reaching their full development within thirty-six to forty-eight hours. Those which grow in long chains usually give an abundant flocculent deposit and leave the liquid clear. The deposit may be in grains, in tiny flocculi, in larger flakes, or in tough, almost membranous masses, the differences depending on the strength of union between the pairs of cocci in the chains. Some of the streptococci growing in long chains, however, cause the broth to become cloudy. This cloudiness may be only temporary or it may be lasting. Those growing in short chains, as a rule, cloud the broth, this cloudiness remaining for days or weeks. A granular deposit appears at the bottom of the tube. An addition of 0.5 to 1 per cent. glucose aids the development of streptococci, but the acid produced tends later to hasten their death and make them lose virulence. A trace of calcium aids the growth. This is best added as a piece of marble, which has the additional advantage of neutralizing some of the acids produced.

**Growth in Ascitic or Serum Bouillon.**—The development in this, which is the best medium for the growth of all streptococci, is more abundant than in plain bouillon. The liquid is usually clouded, and a precipitate occurs after some days, the fluid gradually clearing. The addition of blood serum frequently causes streptococci, growing in short chains in nutrient bouillon, to produce long chains. The reverse is also true, and in the blood all forms are usually found, some, at least, being diplococci or in short chains.

**Effect on Inulin.**—This is not fermented by varieties of the pyogenes type.

**Growth on Solidified Blood Serum.**—This is also an excellent medium for the streptococcus. Tiny, grayish colonies appear twelve to eighteen hours after inoculation.

**Growth in Milk.**—All streptococci grow well in milk. As a rule when growth is luxuriant a marked production of lactic acid with coagulation of the casein occurs.

**Development of Hemolytic Substances.**—Hemolysis is demonstrated within blood agar plates or in fluid containing blood in test-tubes. The plate method is as follows: If 1 c.c. of fresh or defibrinated blood is added to 6 c.c. of melted agar at 40° to 45° C., well shaken, inoculated with characteristic streptococci and poured into a Petri dish there will appear in twelve to twenty-four hours tiny colonies surrounded by clear zones of about  $\frac{1}{4}$  to  $\frac{1}{3}$  inch in diameter.

The tube method is used for quantitative determination of hemolysis.

The titration is made by adding decreasing amounts of a definite culture (eighteen-hour 2 per cent. peptone ascitic broth culture, according to Lyall), to a constant quantity of washed red blood cells (1 c.c. of a 5 per cent. suspension of sheep's red cells, Lyall). The tubes are incubated in water-bath at 37° C. for one hour and readings are then made.

Pneumococci and many streptococci, grouped as *Streptococcus viridans*, which occur together with characteristic forms in the throat, lungs, and elsewhere, on the other hand, produce only narrow zones of a green pigment. Anthony in our laboratory has found that from a streptococcus producing abundant hemolytic substances strains may be obtained, by selecting certain colonies, which fail to make them. She has not been able to obtain from strains producing in first cultures the green pigment only any strains producing hemolytic substances.

**Duration of Life Outside of the Body.**—This is not, as a rule, very great. When dried in blood or pus, however, they may live for several months at room temperature, and longer in an ice-chest, and in gelatin and agar cultures they live for from one week to three months. In order to keep streptococci alive and vigorous, it is best to transplant them frequently. They may be kept alive for a long time in semisolid agar stick cultures at room temperature or in serum or ascitic fluid bouillon in small sealed glass tubes in the ice-chest.

**Resistance to Heat and Chemicals** is given under Disinfection, Part III.

**Non-hemolytic Streptococci.**—Non-hemolytic streptococci, most of which produce a green zone (methemoglobin) on blood agar plates, are not of such active virulence as are the hemolytic type. They are slowly invasive and may produce chronic inflammation of a low grade. The methemoglobin produced by many of the strains in variable amounts is probably a combination of reduction and oxidation processes (Heubner, Cole and Blake). It has no relation to virulence and very little to carbohydrate reactions. Based on cultural and agglutinative reactions this group is apparently heterogeneous.

**Pathogenesis.**—The majority of test animals are not very susceptible to infection by streptococci from tumors, and hence it is difficult to obtain any definite pathological alterations in their tissues through the inoculation into them of cultures of this organism by any of the methods ordinarily practised. White mice and rabbits, under similar conditions, are the most susceptible, and these animals are therefore usually

employed for experimentation. Streptococci, however, differ greatly in the effects which they produce in inoculated animals, according to their animal virulence, which is very different from human virulence. The most virulent, when injected in the minutest quantity into the circulation or into the subcutaneous tissues of a mouse or rabbit, produce death by septicemia. Those of somewhat less virulence produce the same result when injected in considerable quantities. Those still less pathogenic produce septicemia, which is mild or severe, when injected into the circulation; but when injected subcutaneously, they produce abscess or erysipelas. The remaining streptococci, unless introduced in quantities of 20 c.c. or over, produce only a slight redness, or no reaction at all, when injected subcutaneously, and little or no effect when injected directly into the circulation. Many of the streptococci obtained from cases of cellulitis, abscess, empyema, and septicemia belong to this group.

A number of varieties of streptococci have thus been discovered, differing in virulence and in their growth on artificial media; but all attempts to separate them into various classes, even with the use of specific serum, have largely failed, because the differences observed, though often marked, are not constant, many varieties having been found to lose their distinctive characteristics, and even to apparently change from one class to another. A further objection to any of the existing classifications of streptococci, which are based on the manner of growth on artificial culture media, is that it has been impossible to make any which would at the same time give even an approximate idea of their virulence. Experiments have proved that streptococci originally virulent may become non-virulent after long cultivation on artificial media, and, again, that they may return to their original properties after being passed through the bodies of susceptible animals. The peculiar type of virulence which they may acquire tends to perpetuate itself, at least for a considerable time.

One important fact that experience teaches us is that those streptococci which are the most dangerous are those which have come immediately from septic conditions, and the more virulent the case the more virulent the streptococci are apt to be for animals of the same species. There seems also to be a strong tendency for a streptococcus to produce the same inflammation, when inoculated, as the one from which it was obtained; for example, streptococci from erysipelas tend to produce erysipelas, from septicemia to produce septicemia, etc. Streptococci, however, obtained from different sources (abscesses, puerperal fever, erysipelas, etc.) are in many instances capable, under favorable conditions, of producing erysipelas when inoculated into the ear of a rabbit, as has been proved by experiment, provided they possess sufficient virulence.

**Occurrence in Man.**—Streptococci have been found to be the primary cause of infection in the following diseases: Erysipelas, circumscribed and extensive acute abscesses, impetigo, cellulitis (circumscribed as well as diffused), sepsis, puerperal infection, acute peritonitis, angina,

bronchopneumonia, periostitis, osteomyelitis, synovitis, otitis media, mastoiditis, enteritis, irregular cases of rheumatic fever, meningitis, pleurisy, empyema, and endocarditis. Associated with other bacteria in diseases of which they were the specific cause, they have also been found as the secondary infection in many diseases, such as in pulmonary tuberculosis, bronchopneumonia, septic diphtheria, and diphtheritic scarlatina.

In cases of septic thrombus of the lateral sinus following mastoiditis there is almost certainly a streptococcus septicemia. Libman has shown that an examination of the blood may be useful in diagnosis.

In diphtheritic false membranes this micrococcus is very commonly present, and is frequently the source of deeper infection, such as abscesses and septicemia; and in certain cases accompanied by a diphtheritic exudation, in which the Löffler bacillus has not been found by competent bacteriologists, it seems probable that the *Streptococcus pyogenes*, alone or with other pyogenic cocci, is responsible for the local inflammation and its results. These forms of so-called diphtheria, as first pointed out by Prudden, are most commonly associated with scarlatina and measles, erysipelas, and phlegmonous inflammation, or occur in individuals exposed to these or other infectious diseases. So uniformly are long-chained streptococci present in the pseudomembranes of patients sick with scarlet fever, that many investigators have suspected a special variety of them to be the cause of this disease. The same is true for smallpox. Many varieties are regularly found, however, in the throat secretion of healthy individuals (in 100 examinations by us we found long-chained streptococci in 83, and probably could have found them in some of the others by longer search). Their abundance in scarlet fever and smallpox is most probably due to their increase in the injured mucous membrane and entrance into the circulation when the protective properties of the blood have been lowered.

Recently, Rosenow and his co-workers have claimed that a variety of non-hemolyzing streptococci is the cause of poliomyelitis (see Filtrable Viruses).

**Septic Sore Throat.**—Streptococcus infection of the throat appears at times as a severe epidemic. Most of these epidemics have been traced to the milk supply. As Smith, Brown, Krumwiede and Valentine and others have shown, these streptococci probably always come originally from a septic human throat. Streptococci of human origin may invade the milk ducts and multiply in the udder without causing any physical signs of mastitis. The bovine streptococci normally producing mastitis (Mathers) have no relation to septic sore throat. The local infection is usually accompanied by severe general symptoms and by suppurative foci elsewhere.

**Occurrence in Animals.**—Besides streptococci similar to those in man, animals are infected by strains that are negative to Gram and fluidify gelatin. Udder infections of the cow and glandular diseases of the horse are frequently due to these. The streptococcic inflammations in animals are almost as frequent and serious as they are in man.

**Effect on Tumors.**—Fehleisen inoculated cultures, obtained in the first instance from the skin of patients with erysipelas, into patients in the hospital suffering from inoperable malignant growths—lupus, carcinoma, and sarcoma—and he obtained positive results, a typical erysipelatous inflammation having developed around the point of inoculation after a period of incubation of from fifteen to sixty hours. This was attended with chilly sensations and an elevation of temperature. Persons who had recently recovered from an attack of erysipelas frequently proved to be immune. These experiments were undertaken on the ground that malignant tumors had previously been found to improve or entirely disappear in persons who had recovered from accidental erysipelas. This fact was therapeutically applied to the treatment of malignant tumors. Then the mixed toxins of the streptococcus and *B. prodigiosus* were given, and it became apparent that the toxins of the latter organism were much the more important. In some cases of inoperable sarcoma this method met with considerable success (Coley). The injections cause severe reactions.

**Production of Toxic Substances.**—There is no doubt that the streptococcus causes fever, general symptoms of intoxication, and death by means of toxic substances which it forms in its growth; but we know very little about these substances or how they are produced. The cell substance of streptococci possesses only slight toxicity. Ruediger has shown that a specific streptolysin is formed which produces a true antibody. The poisons while partly extracellular are mostly contained in the cell substance. Heat destroys a portion of them. They appear to attack especially the red blood cells, and this hemolytic action seems to be to some degree in proportion to the virulence of the organism.

**Susceptibility to Streptococcus Infection.**—As with the ever-present staphylococci, whose virulence, as we have seen, is usually slight, the streptococci are more likely to invade the tissues, forming abscesses or erysipelatous and phlegmonous inflammation in man when the standard of health is reduced from any cause, and especially when by absorption or retention various toxic organic products are present in the body in excess. It is thus that the liability to these local infections, as complications of operations or sequelæ of various specific infectious diseases, in the victims of chronic alcoholism, and constitutional affections, etc., are to be explained. It seems established that the absorption of toxic products formed in the alimentary canal as a result of the ingestion of improper food, or in consequence of abnormal fermentative changes in the contents of the intestine, or from constipation predispose to infection.

**Immunity.**—In none of the streptococcus inflammations do we notice much apparent tendency to the production of immunizing and curative substances in the blood by a single infection.

Several general infections usually progress to a fatal termination after a few days, weeks, or months. It is true, however, that cases of erysipelas, cellulitis, and abscess, after periods varying from a few days to months, tend to recover, and to a certain extent, therefore, we

may assume that protective agents have been produced. In these cases, however, we know from experience that faulty treatment, by lessening the local or general resistance, would, as a rule, cause the subsiding infection again to progress perhaps even to a more serious extent than the original attack. Koch and Petruschky tried a most interesting experiment. They inoculated cutaneously a man suffering from a malignant tumor with a streptococcus obtained from erysipelas. He developed a moderately severe attack, which lasted about ten days. On its subsidence they reinoculated him; a new attack developed which ran the same course and over the same area. This was repeated ten times with the same results.

This experiment proved that in this case, at least, the immunizing substances produced by repeated attacks of erysipelas were insufficient to make the tissues and lymph sufficiently bactericidal to prevent infection.

The severe forms of infection, such as septicemia following injuries, operations, and puerperal infections, show little tendency to be arrested after being well established. Having in mind the above facts, let us consider the results already obtained in the experimental immunization and treatment of animals and men suffering from or in danger of infection with streptococci. Knorr succeeded in producing a moderate immunity in rabbits against an intensely virulent streptococcus by injections of very slightly virulent cultures. Marmorek was the first to attempt the production of a curative serum on a large scale.

**Influence of Serum from Immunised Animals upon Streptococcus Infections in Other Animals.**—In the table are given the results following the injection of small amounts of a serum which represents in immunizing value what about one-third of the horses are able to produce when given in gradually increasing doses a living, virulent streptococcus. In the following experiments the serum and culture were injected subcutaneously into rabbits, into some inoculated after mixing and into others separately and on opposite sides of the body.

SHOWING STRENGTH OF AVERAGE GRADE OF ANTISTREPTOCOCCIC SERUM  
GIVEN BY SELECTED HORSES AFTER SIX MONTHS OF INJECTION OF  
SUITABLE AMOUNTS OF LIVING STREPTOCOCCI.

	Weight of rabbit.	Amounts inoculated. Serum culture.	Results.	Autopsy.
1. Inoculated together . .	1430	0.25 c.c. 0.01 c.c.	Lived	
2. Inoculated together . .	1350	0.125 c.c. 0.01 c.c.	Lived	
3. On opposite sides . .	1770	0.1 c.c. 0.01 c.c.	Lived	
4. On opposite sides . .	1630	0.1 c.c. 0.01 c.c.	Lived	
Controls:				
1. Rabbits injected with cul- ture only.	1750	.... 0.001 c.c.	Died in 4 days	Streptococcic infection.
2. Rabbits injected with cul- ture only.	1870	.... 0.001 c.c.	Died in 24 hrs.	Streptococcic infection.

The above results have been repeatedly obtained, and are absolutely conclusive that the serum of properly selected animals, which have been repeatedly injected with living streptococci in suitable doses,

possesses bactericidal properties upon the same streptococcus when it comes in contact with it within the bodies of animals.

**Polyvalent Serum.**—Results of investigators show that the majority of hemolytic streptococci met with in cellulitis, erysipelas, and abscess will be influenced by the same serum. In order that the serum may have specific antibodies for the variety of streptococci causing each separate infection, each horse is now injected with a large number of different varieties of streptococci. This serum will not be as good as if made by the streptococcus infecting the treated case, but will be fairly efficient for all cases. The non-hemolyzing streptococci obtained from cases of pneumonia and endocarditis and other exceptional infections are apt to have individual characteristics. Here a polyvalent serum is of little value.

**Dosage.**—It is found that the immune bodies to be effective must be in sufficient concentration. The intravenous injections are usually 100 c.c. for an adult.

**Preparation of the Serum.**—The preparation of antiserums is given in Part III under Applications of Serum Therapy.

**Stability of the Serum.**—It is fairly stable but, after several months, the serum loses some of its protective value. It should be kept in a cool and dark place.

**Standardization of the Value of the Serum.**—There is at present no satisfactory way. The value of the serum is sometimes measured by the amount required to protect against a multiple of a fatal dose of a very virulent streptococcus of the same type as the one used to inject the horses. The dose is usually a thousand times the average fatal amount of a very virulent streptococcus.

Other methods of standardization, such as the estimation of the amount of opsonins or agglutinins present, are also used but are not as conclusive.

**Therapeutic Results.**—To estimate the exact present and future value of antistreptococcus serum is a matter of the utmost difficulty. Many of the cases reported are of little or no help, because, no cultures having been made, we are in doubt as to the nature of the bacterial infection.

In the cases of puerperal fever, erysipelas, and wound infection that we have seen, the apparent results under the treatment have not been uniform. We have frequently observed favorable results which appeared to be due to the serum when doses of 50 to 100 c.c. were given intravenously.

In a number of cases of septicemia in which for days chills had occurred daily they ceased absolutely or lessened under daily doses of 20 to 50 c.c. The temperature, though ceasing to rise to such heights, did not average more than one or two degrees lower than before the injections. In some cases the serum treatment was kept up for four weeks. Some cases convalesced; others after a week or more grew worse and died. In some cases the temperature fell immediately upon giving the first injection of serum, and after subsequent injections remained

normal, and the cases seemed greatly benefited. As a rule, in these cases no streptococci or any other organisms were obtained from the blood. In bronchopneumonia due to streptococci and in the mixed infections accompanying laryngeal diphtheria, tonsillitis, smallpox, and phthisis, we have seen little effect.

The results obtained here in New York by both physicians and surgeons in streptococcus infections have not, on the whole, been very encouraging.

In some of the cases where apparently favorable results were obtained other bacteria than streptococci were found to be the cause of the disease. We believe that the following conclusions will be found fairly accurate:

The serum will in animals limit an infection already started if it has not progressed too far. The apparent therapeutic results in cases of human streptococcus infection are variable. In some cases the disease has undoubtedly advanced in spite of large injections, and here it has not seemed to have had any effect. In other cases good observers rightly or wrongly believe they have noticed great improvement from it. Except rashes, few have noticed deleterious results, although very large doses have been followed in several instances, for a short time, by albuminous urine.

In suitable cases we are warranted, we believe, in trying it, but we should not expect very striking results.

For our own satisfaction, and to increase our knowledge, we should always have satisfactory cultures made when possible, and the streptococci, if obtained, tested with the serum used in the treatment. In the cases where we want most to use the serum, such as puerperal fever, septicemia, ulcerative endocarditis, etc., we find that it is very difficult to make a bacteriological diagnosis from the symptoms, and in over one-half of the cases even the bacteriological examination carried out in the most thorough way will fail to detect the special variety of bacterium causing the infection. This is often a great hindrance to the proper use of curative antistreptococcic serum, for it, of course, has no specific effect upon the course of any infection except that due to the streptococcus and the full effect only on its own type.

Care should be taken to get the most reliable serum; much on the market is worthless, and as it is weak, and the testing for strength is difficult or impossible, full doses (50 to 100 c.c.) of serum should be given if the case is at all serious, for the dose is limited only by the amount of horse serum which we feel it safe to give, not because we have given sufficient protective substance. Intravenous injections give better results than those given subcutaneously. Studdiford has obtained good results by adding to the intravenous injection the packing of the septic uterus with gauze impregnated with the serum.

**Scarlet Fever.**—In Vienna for some years the serum of horses treated at each injection with a number of strains of streptococci derived from scarlet-fever cases has been used in this disease. The serum given in large doses of 100 to 200 c.c. has apparently given good results in about



half of those treated. It is only used in severe cases. Moser has chiefly advocated its use. One of us had the opportunity of looking over the histories of his cases. Although left in doubt as to its value, it appears to us as worth a trial. Our own results and Nicoll's, in cases occurring in the Health Department hospitals, have been rather favorable.

**Streptococcus Vaccine.**—The preparation and use of streptococcus vaccines is given in Part III, under Practical Applications of Vaccines.

**Complement-fixation.**—The method and results of this test have been considered in Part I, p. 199.

**Bacteriological Diagnosis.**—Streptococci, using the name in a broad sense, can often be demonstrated microscopically by simply making a smear preparation of the suspected material and staining with methylene-blue solution or diluted Ziehl's stain. In order to demonstrate them microscopically in the tissues the sections are best stained by Kühne's methylene-blue method. In all cases, even when the microscopic examination fails, the cocci may be found by the use of culture media, such as broth or plated agar at 37° C. To obtain them from a case of erysipelas it is best to excise a small piece of skin from the margin of the erysipelatous area in which the cocci are most numerous; this is crushed up and part of it transferred to ascitic or serum bouillon, and part is streaked across freshly solidified agar in a Petri dish on which a drop of sterile rabbit's blood has been placed. Both are kept in the incubator at 37° C.

In septicemia the culture method is always required to demonstrate the presence of streptococci, as the microscopic examination of specimens of blood is not sufficient. For this purpose from 10 to 15 c.c. of the blood should be drawn from the vein of the arm aseptically by means of a hypodermic needle, and to each of three tubes containing 10 c.c. of melted nutrient agar kept at about 43° C., 1 c.c. of blood is added. After thorough mixing, the contents are poured into Petri dishes. The remainder is added to several flasks containing 100 c.c. of nutrient broth, in order to produce a development of the cocci, which are found in small numbers in the blood. Petruschky is of the opinion that the cocci can be best shown in blood by animal inoculation. Having withdrawn from the patient 10 c.c. of blood by means of a hypodermic syringe, under aseptic precautions, he injects a portion of this into the abdominal cavity of a mouse, while the other portion is planted in bouillon. Mice thus inoculated die from septicemia when virulent streptococci are present in only very small numbers in the blood. If a successful inoculation takes place we can, through the absence or presence of the development of capsules, often differentiate between the pneumococcus and the streptococcus. Cultures may fail to do this. The development of a wide, clear zone about the colonies (upon blood agar), without a development of green pigment, indicates that the streptococci belong to the pyogenes type. The absence of a definite zone and the development of a green color indicates that they are pneumococci, or streptococci of the viridans type. The growth in the Hiss inulin serum medium will generally differentiate between the two, as

the pneumococci usually coagulate the serum, while the great majority of streptococci do not. Agglutination may also be tried but this would only differentiate certain strains. The morphological and cultural characteristics of the streptococcus give us, unfortunately, no absolute knowledge as to the influence which the protecting serum will have. The actual test is here our only method. The detection of the streptococcus in the blood is in itself an unfavorable prognostic sign.

The blood cultures in many cases of supposed septicemia give no results, for many of these cases develop their symptoms and even die from the absorption of toxins from the local infection, such as an amputation wound or an infected uterus or peritoneum, and the bacteria never invade the blood. When we get negative results we are, as a rule, utterly unable to test the case with curative serums with any accuracy, for the sepsis may be due to either the streptococcus, colon bacillus, staphylococcus, or a number of other pathogenic varieties of bacteria.

In mixed infections when the streptococci present may not be isolated from original plates, plates made from an eighteen-hour culture in suitable broth may contain many isolated colonies.

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## CHAPTER XVII.

### THE DIPLOCOCCUS OF PNEUMONIA (PNEUMOCOCCUS, STREPTOCOCCUS PNEUMONIÆ, MICROCOCCUS LANCEOLATUS).

#### THE DIPLOCOCCUS OF PNEUMONIA.

THE diplococcus of pneumonia was observed in 1880 almost simultaneously by Sternberg and Pasteur in the blood of rabbits inoculated with human saliva. In the next few years Talamon, Friedländer, A. Fränkel, Weichselbaum, and others subjected this microorganism to an extended series of investigations and proved it to be the chief etiological factor in the production of lobar or croupous pneumonia in man. The relationship of this organism to the streptococcus group is spoken of in the preceding chapter.

The outcome of the various investigations proved that the acute lung inflammations, especially when not of the frank lobar pneumonia type, are not excited by a single variety of microorganism, and that the bacteria involved in the production of pneumonias are also met with in inflammations of other tissues.

In any individual pneumonic inflammation it is also found that more than one variety of bacteria may be active, either from the start or as a later addition to the original primary infection.

Among all the microorganisms active in exciting pneumonia, the diplococcus of pneumonia is by far the most common, being almost always present in primary lobar pneumonia and as frequently as any other germ in acute bronchopneumonia and metastatic forms. Besides the different varieties of pneumococci the following bacteria are capable of exciting pneumonia: *Streptococcus pyogenes*, *Staphylococcus pyogenes*, *Bacillus pneumoniae*, *Bacillus influenzae*, *Bacillus pestis*, *Bacillus diphtheriae*, *Bacillus typhi*, *Bacillus coli*, and the *Bacillus tuberculosis*. Since the varieties of bacteria exciting acute pneumonia, with the exception of the pneumococcus, are met with more frequently in other inflammations and have been described elsewhere, they will only be noticed in this chapter so far as their relation to pneumonia demands.

**Morphology.**—Typically, the pneumococcus occurs as spherical or oval cocci, usually united in pairs, but sometimes in longer or shorter chains consisting of from three to six or more elements and resembling the streptococcus. The cells, as they commonly occur in pairs, are somewhat oval in shape, being usually pointed at one end—hence the name *lanceolatus* or lancet-shaped. When thus united the junction, as a rule, is between the broad ends of the oval, with the pointed ends turned outward; but variation in form and arrangement of the

cells is characteristic of this organism, there being great differences according to the source from which it is obtained. As observed in the sputum and blood it is usually in pairs of lancet-shaped elements, which are surrounded by a capsule. (See Fig. 106.) When grown in fluid culture media longer or shorter chains are frequently formed, which can scarcely be distinguished from chains of certain streptococci, except that, as a rule, the length of the chain is less and the pairs of diplococci are farther apart. In cultures the individual cells are almost spherical in shape, and except in certain varieties are rarely surrounded by a capsule. (See Fig. 107.) The pneumococcus is by some classed as a streptococcus. Rosenow claims that a typical pneumococcus may be easily changed into a typical streptococcus. (See preceding chapter.)

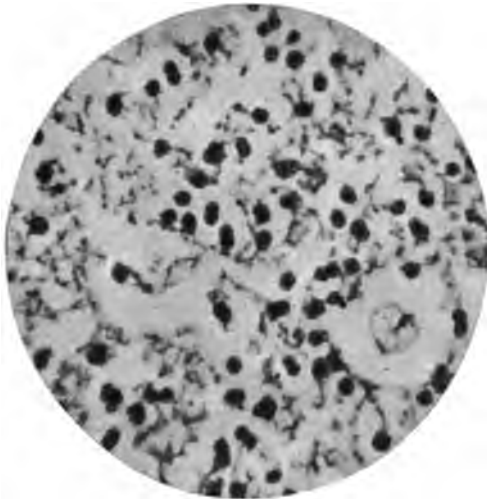


FIG. 106.—Diplococcus of pneumonia from blood, with surrounding capsule stained by method of Hiss.

The capsule is best seen in stained preparations from the blood and exudates of fibrinous pneumonia or from the blood of an inoculated animal, especially the mouse, in which it is commonly, though not always, present. It is seldom seen in preparations from cultures unless special media are employed. Flagella are not present.

**Staining.**—It *stains* readily with ordinary aniline colors; it is not decolorized after staining by Gram's method. The capsule may be demonstrated in blood or sputum by the methods given (p. 78).

**Biology.**—It grows equally well with or without oxygen; its parasitic nature is exhibited by the short range of temperature at which it usually grows—viz., from 25° to 42° C.—best at 37° C. In the cultivation of this organism neutral or slightly alkaline media should be employed. The organism when freshly isolated grows feebly on the serum-free culture media ordinarily employed for the cultivation of bacteria—viz., on nutrient agar and gelatin, in bouillon. The best medium for its

growth is a mixture of one-third human or animal blood serum or ascitic or pleuritic fluid and two-thirds bouillon, or nutrient agar streaked with human, horse, or rabbit blood.

**Growth on Agar.**—Cultivated on plain nutrient agar, after twenty-four to forty-eight hours at 37° C., the deep colonies are hardly visible to the eye. Under the microscope they appear light yellow or brown in color and finely granular. The surface colonies are larger, equaling in size those of streptococci, but are usually more transparent. If blood serum or ascitic fluid is added to the agar the individual colonies are larger and closer together, and the growth is more distinct in consequence and of a grayish color. The surface colonies are almost circular in shape under a magnification of 60 diameters, finely granular in structure, and may have a somewhat darker, more compact centre, surrounded by a paler marginal zone. With high magnification cocci in twos and short rows often distinctly separated are seen at the edges.



FIG. 107.—Pneumococcus from bouillon culture, resembling streptococcus.

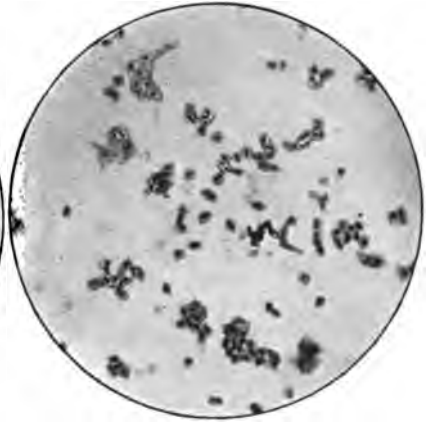


FIG. 108.—Pneumococci stained for capsule by Huntoon's method. (Huntoon.)

**Growth on Blood Agar.**—The colonies on blood agar are greenish and no hemolysis is present. This is a useful characteristic in isolating pneumococci from sputum, although many streptococci give similar colonies. Methemoglobin is produced in broth cultures as shown on addition of freshly washed red corpuscle suspensions.

**Growth on Blood Serum.**—The growth on Löffler's blood-serum mixture is very similar to that on agar, but somewhat more vigorous and characteristic, appearing on the surface as a delicate layer of dew-like drops.

**Growth in Bouillon.**—In bouillon, at the end of twelve to twenty-four hours in the incubator, a slight cloudiness of the liquid will be found to have been produced. On microscopic examination cocci can be seen to be arranged in pairs or longer or shorter chains. After one or two transplantations the pneumococci frequently fail to grow.

**Growth in Milk.**—It grows readily in milk causing coagulation with the production of acid, though coagulation is not constant with some forms intermediate between the streptococcus and pneumococcus.

**Growth on Gelatin.**—The growth on gelatin is slow, if there is any development at all, owing to the low temperature—viz., 24° to 27° C.—above which even the most heat-resistant gelatin will melt. The gelatin is not liquefied.

**Special Media.**—When cultures are grown on serum-free media the vitality of some cultures may indeed be indefinitely prolonged; but after transplantation through several generations it is found that the cultures begin to lose in virulence, and that they finally become non-virulent. In order to restore this virulence, or to keep it from becoming attenuated, it is necessary to interrupt the transplantation and pass the organism through the bodies of susceptible animals.

The vitality is prolonged and the virulence less rapidly lost if serum ascitic fluid or blood is present in the medium. Serum or ascitic broth, serum semi-solid (stab cultures) or blood-streaked agar are the most satisfactory media for the preservation of cultures.

**Action of Bile and Bile Salts.**—If 0.1 c.c. of rabbits' bile be added to 1 or 2 c.c. of a broth culture of pneumococci the culture becomes clear due to the dissolving of the cocci. A 10 per cent. solution of sodium taurocholate has the same action. Streptococci are not dissolved by bile. Serum and glucose interfere with the reaction.

**Hiss Serum-water with and without Inulin.**—These are very useful. The inulin is fermented by typical pneumococci with coagulation of the serum, while most streptococci fail to ferment the inulin. This medium is therefore of considerable diagnostic value.

**Calcium Broth with or without Dextrose.**—The addition of a small piece of marble to each tube of broth is the most satisfactory way of preparing it. Marble broth for this purpose was suggested independently by Bolduan and Hiss as very satisfactory for growth.

**Resistance to Light and Drying** (see Media).—On artificial culture media the pneumococci tend to die rapidly. This is partially due to the acid produced by their growth. In sputum they live much longer.

Pneumonic sputum attached in masses to clothes, when dried in the air and exposed to diffuse daylight, retains its virulence, as shown by injection in rabbits, for a period of nineteen to fifty-five days. Exposed to direct sunlight the same material retains its virulence after but a few hours' exposure. This retention of virulence for so long a time under these circumstances is accounted for by the protective influence afforded by the dried mucoid material in which the micrococci were embedded. Guarnieri observed that the blood of inoculated animals, when rapidly dried in a desiccator, retained its virulence for months; and Foá found that fresh rabbit blood, after inoculation and cultivation in the incubator for twenty-four hours, when removed at once to a cool, dark place, retained its virulence for sixty days. There are many conditions, therefore, in which the virulence of the micrococcus is retained for a considerable length of time; the fine spray expelled in coughing and loud speaking that remains suspended in the air soon dries so completely that probably no pneumococci survive after two hours. The action of chemical disinfectants is given in Part III under Disinfection.

**Attenuation of Virulence.**—The loss of virulence which occurs when the micrococcus is transplanted through several generations in culture fluid containing no blood has already been referred to. An attenuation

of virulence, it has been claimed, takes place also spontaneously in the course of pneumonia. This attenuation is probably only apparent. If a little sputum is taken at different periods in the disease and planted in ascitic bouillon the resultant cultures do not vary greatly in virulence.

**Restoration and Increase of Virulence.**—The simplest and perhaps the most reliable method of restoring lost virulence for any susceptible animal is by passage through the bodies of highly susceptible animals of the same species. Growth in fresh blood also increases it for the homologous animal.

**Maintenance of Virulence.**—This is best done by drying the spleen of an infected animal in a dessicator. The spleen should be removed just before or immediately after death. The virulence is preserved in this way for a month or longer.

**Toxin Production.**—We have little exact knowledge upon the nature of the substances produced by or through the growth of the pneumococci in animal tissues or artificial media. Rosenow showed that the autolysis of virulent pneumococci in NaCl solution brings into the solution a group of substances which inhibits the action of the pneumococco-opsonin. Such extracts are also toxic for animals. Pneumococci dissolved in bile uniformly yield a toxic product. A hemotoxin is present in the bile extracts and can also be extracted by other methods.

**Occurrence in Man during Health.**—It is probable that in crowded communities the pneumococcus is present on the mucous membranes of most persons. We have found it generally present not only in the throats of persons living in New York City, but also in those of persons living on farms and in the Adirondack Mountains. It is commonly present only on the mucous membranes of the bronchi, trachea, pharynx, and nostrils. The healthy lung seems to be generally free from it. The type found is usually one of the less virulent members of Group IV (see below). Carriers of the more virulent types also occur, usually due to contact with a case of pneumonia.

**Pathogenicity in Man.**—Pneumococci, characteristic or atypical, are present in fully 95 per cent. of characteristic cases of lobar pneumonia. Usually no other bacteria are obtained from the lungs. Atypical cases usually show the same conditions, but they may be due to streptococci, influenza bacilli, etc. The more recent the infection the greater is the number of bacteria found in the diseased lung area. As the disease progresses these decrease in number until finally at the crisis they disappear from the tissues, though at this time and long after convalescence they may be present in the sputum. In atypical forms of pneumonia they may remain longer in the tissues, and in walking pneumonia they may be absent in the original centres of infection or present only as attenuated varieties, while the surrounding, newly formed foci may contain fully virulent cocci. It has been shown by Netter that more than one-half of the cases of bronchopneumonia, whether primary or secondary to some other disease, as measles and diphtheria, both in children and adults, are due to the diplococcus of pneumonia. Others, such as Pearce, have found other microorganisms, especially the strep-

tococci, in the majority of cases. These findings will be considered at the end of the chapter.

The pneumococci are found partly in the alveoli and bronchioles of the inflamed lung and partly in the lymph channels and blood capillaries. Most of the organisms are found free, but a few are found in the leukocytes. Through the lymph channels they find their way to the pleura and to adjacent lymph glands. From the capillaries they find their way to the general blood current, and thus to distant parts of the body. In about 20 per cent. of cases the pneumococci are so abundant that they can be found in cultures made from 5 to 10 c.c. of blood. In a number of instances the fetus has been found infected. The pneumococci are also responsible for:

**Inflammations Complicating Pneumonia.**—In every case of lobar pneumonia and in most cases of bronchopneumonia, pleurisy is developed, which is excited by the same microorganism that was predominant in the pneumonia. With pneumococci the exudate is usually moderate and of a fibrinous character, but may be more abundant and of a sero-fibrinous or purulent character. When the pleurisy is marked it is more apt to continue after the cessation of the pneumonia. Pleurisy due to pneumococci is more apt to go on to spontaneous recovery than that due to streptococci or staphylococci.

The most frequent pneumococcic infections next to pleurisy, following a pneumonia, are those of the middle ear, pericardium, endocardium, and meninges, and these not infrequently arise together. Pneumococcic inflammations of the heart valves are apt to be followed by extensive necrosis and growth of vegetations. In these cases pneumococci can sometimes be found in the blood for many weeks. Pericarditis due to pneumococci is a frequent complication, but is usually very slightly developed. Meningitis due to pneumococci may be either fibrinous or purulent or both and is apt to be secondary to otitis, mastoiditis, or pneumonia. Arthritis, peri-arthritis, and osteomyelitis are rarer complications of a pneumococcic pneumonia. Besides moderate parenchymatous inflammation of the kidney, which occurs in most cases of pneumonia, well-marked inflammation may occur in which pneumococci exist in the kidney tissues in large numbers.

The presence of pneumococci in the blood after death has been amply proved by numerous investigations. In many instances, they have been recovered from the blood during life. Lambert, as a rule, found them in all fatal cases twenty-four to forty-eight hours before death. The conveyance of the infective agent by means of the blood and the lymph to all parts of the body explains the multiplicity of the affections complicating a pneumonia, which are caused by this micrococcus; and not only the secondary, but also the primary diseases, as of the brain and meninges, may be explained in the same way.

**Presence in Inflammatory Process Not Secondary to Pneumonia.**—It is now known that the pneumococcus may infect and excite diseases in many tissues of the body independent of any preliminary localization in the lung. As a rule these processes are acute and usually run a



shorter and more favorable course than similar inflammations due to the streptococci.

The most frequent primary lesions excited by the pneumococcus after lobar pneumonia, bronchopneumonia, and bronchitis are probably meningitis, otitis media with its complicating mastoiditis, endocarditis, pericarditis, rhinitis, tonsillitis, conjunctivitis, and keratitis; septicemia, arthritis, and osteomyelitis; inflammations of the epididymis, testicles, and Fallopian tubes; peritonitis, etc.

Pneumococcic peritonitis and appendicitis are not so very frequent. The exudate is usually seropurulent.

Conjunctivitis due to pneumococci frequently occurs in epidemic form and is frequently associated with rhinitis.

From statistics collected by Netter the following percentages of diseases were caused by the pneumococcus:

Pneumonia . . . . .	65.9 per cent. in adults.
Bronchopneumonia . . . . .	15.8 " "
Meningitis . . . . .	13.0 " "
Empyema . . . . .	8.5 " "
Otitis media . . . . .	2.4 " "
Endocarditis . . . . .	1.2 " "

In 46 consecutive pneumococcus infections in children there were:

Otitis media . . . . .	29 cases.
Bronchopneumonia . . . . .	12 "
Meningitis . . . . .	2 "
Pneumonia . . . . .	1 case.
Pleurisy . . . . .	1 "
Pericarditis . . . . .	1 "

The pneumococcus and streptococcus are the two most frequent organisms found in otitis media. The cases due to the pneumococcus are apt to run the shorter course, but have a tendency to spread to the meninges and cause a meningitis. The pneumococci may also find their way into the blood current. This usually follows after sinus thrombosis.

In bronchitis the pneumococcus is frequently met alone or in combination with the streptococcus, the influenza bacillus, or other bacteria.

In certain epidemics pneumococcic bronchitis and pneumonia simulate influenza very closely and cannot be differentiated except by bacteriological examinations.

Primary pneumococcic pleurisy is frequent in children: it is very often purulent, but may be serous or serofibrinous. Its prognosis is better than that in cases due to other organisms. Frequently we have streptococci and staphylococci associated with the pneumococci.

**Pathogenesis in Lower Animals.**—Most strains of the *Micrococcus lanceolatus* are moderately pathogenic for numerous animals; mice and rabbits are the most susceptible, indeed some strains are intensely virulent for these animals, while guinea-pigs and rats are much less susceptible. Pigeons and chickens are refractory. In mice and rabbits the subcutaneous injection of small or moderate quantities of pneu-

monic sputum in the early stages of the disease, or of a twenty-four-hour ascitic broth culture from such sputum, or of a pure, virulent ascitic broth culture of the micrococcus, usually results in the death of these animals in from twenty-four to forty-eight hours. The course of the disease produced and the postmortem appearances indicate that it is a form of septicemia—what is known as sputum septicemia. After injection there is loss of appetite and great debility, and the animal usually dies some time during the second day after inoculation. The postmortem examination shows a local reaction, which may be of a serous, fibrinous, hemorrhagic, necrotic, or purulent character; or there may be combinations of all of these conditions. The blood of inoculated animals immediately after death often contains the micrococci in very large numbers. For microscopic examination they may be obtained from the blood, and usually from pleural and peritoneal exudates when these are present.

True localized pneumonia does not usually result from subcutaneous injections into susceptible animals, but injections made through the thoracic walls into the substance of the lung may induce a typical fibrinous pneumonia. This was first demonstrated by Talamon, who injected the fibrinous exudate of croupous pneumonia, obtained after death or drawn during life from the hepatized portions of the lung, into the lungs of rabbits. Wadsworth showed that by injecting virulent pneumococci into the lungs of rabbits which had been immunized, a typical lobar pneumonia was excited, the bactericidal property of the blood being sufficient to prevent the general invasion of the bacteria. Pneumonia may be produced in dogs, and in rabbits less easily, by intratracheal injections.

**Varieties of the Pneumococcus.**—As among all other microorganisms minutely studied, different strains of pneumococci show quite a wide range of variation in morphology and virulence. Some of the variations are so marked and so constant that they make it necessary to recognize several distinct varieties of the pneumococcus, and to class as pneumococci certain varieties which have before this been classed as streptococci—*e. g.*, the so-called *Streptococcus mucosus capsulatus* (*Streptococcus mucosus* Schottmüller)—when first isolated from pneumonic exudate or elsewhere, and planted on artificial culture media containing serum, grows as a rounded coccus with a small dense distinct capsule, principally in short or medium chains; it produces a large amount of mucus-like zoöglea, forming very large spreading colonies; it promptly coagulates fluid-serum media containing inulin. It is also very virulent for mice, but only moderately virulent for rabbits. After a number of culture generations on ordinary nutrient agar it apparently loses most of these characteristics. It then grows in small colonies principally as naked diplococci which may be elongated and pointed, produces no zoöglea, and loses most of its virulence for mice and rabbits. It still coagulates inulin-serum media, and when transferred to serum media regains its former morphological characteristics. For these reasons we consider this organism a distinct variety of the pneumo-

coccus. This variety of pneumococcus has been isolated by us from the lungs after death following lobar pneumonia, out of twenty consecutive autopsies, as the only organism present twice, and with another variety of pneumococcus once. Together with other varieties it was isolated from four out of twenty specimens of pneumonic sputum, and from sixty specimens of normal throat secretion five times. In 1905 Park and Williams showed that this variety should be placed with the pneumococci under the common name *pneumococcus mucosus*.

**Agglutination Reactions.**—That agglutinins are produced in animals by the injection of pneumococci was shown by Neufeld, Clairmont, and others. It has since been shown that this test may be used as a means of diagnosis. Neufeld, Collins, Cole and Dochez and others have shown that certain pneumococci may be grouped according to serum reactions. According to Cole and Dochez, the groups based upon their agglutination reactions are as follows: Group I and II, typical pneumococci; Group III, pneumococcus mucosus; Group IV, heterogeneous strains. Each strain in the last group seems independent as far as serum reactions are concerned; it is really not a group but rather an assembling together of isolated strains. More recently Avery has shown that occasionally pneumococci occur which are related to Group II in that the serum of a type organism protects animals against infection by these subtypes, at least to a very considerable degree, but the reverse, that a serum produced by one of them protects against the others or the standard Type II, has not been demonstrated. More than one subgroup evidently exists. Variations in the agglutinative characteristics of pneumococci can be induced. Passage through a susceptible animal causes a return of its type reaction.

The following table by Cole gives the types he found in pneumonia in a series of cases occurring in New York City, and the relative mortality of the cases according to the infecting type:

Infecting type.	Cases.	Strain, per cent.	Deaths.	Mortality, per cent.
I . . . . .	34	47	8	24
II . . . . .	13	18	8	61
III . . . . .	10	14	6	60
IV . . . . .	15	21	1	7
Total . . . . .	72	100	23	32

In different localities, and at different times in the same locality, the type percentage will probably vary greatly and the mortality also varies somewhat.

Wollstein and Benson found that Group IV occurred more frequently in the pneumonias of children and that the mortality due to this group was high (40 per cent.). Only a limited number of observations are available on the groups occurring in meningitis. Valentine gives the following series: I, 2 cases; II, 7 cases; III, 2 cases; IV, 2 cases. Total, 13.

Pneumococci taken from normal throats or from slight inflammation fall usually into Group IV among the heterogeneous strains. The strains

belonging to Group I are widely scattered, being found not only throughout the United States but also in Europe.

**Rapid Method for Determination of Infecting Type in Sputum.**—The following method is employed at the Rockefeller Institute Hospital: The sputum coughed up from the lung is injected intraperitoneally into a mouse. After four or five hours the mouse is killed and the peritoneal cavity washed out with salt solution. The washings are then centrifuged slowly to throw down the fibrin and cells and the supernatant fluid is then drawn off and centrifuged to throw down the cocci. The sedimented cocci are then suspended in saline and mixed in equal parts with known Types I and II serums respectively, and the presence or absence of agglutination determined after incubation for one or two hours. Type III can usually be diagnosed by the mucoid character of the peritoneal exudate.

With cultures obtained from other sources, broth cultures free of serum are employed for agglutination.

**Immunity.**—Following an attack of pneumonia some immunity is established, but this lasts only a short time. After successive injections of gradually increasing doses of virulent pneumococci into certain animals (horse, sheep, goat, rabbit), a serum of protective and some curative power in experimental animals is obtained. The mode of action of this serum is still the subject of study. According to Wright, Neufeld, and others, its activity is due primarily to the opsonins. The mechanism of the crises in pneumonia is not understood, but the presence of increased opsonins and other protective substances at this time suggests that it is a phenomenon dependent upon the increase of these substances and associated cellular activity.

*Serums and Vaccines* are given in Part III under Applied Therapy.

**Chemotherapy.**—Morgenroth and Levy showed that ethylhydrocuprein (optochin), a derivative of hydroquinone, was of value in the treatment of experimental pneumococcus infections. This substance has an almost specific action on pneumococci and *in vitro* its greater action on the pneumococcus group serves to separate this group from the related cocci.

It acts even in dilutions as great as 1 in 1,000,000. It has a protective and curative action in guinea-pigs and mice infected with pneumococci. Small doses of optochin increase considerably the protective power of the type homologous antipneumococcus serum in experimental animals (Moore).

The pneumococcicidal action of the serum after administration of the drug was studied in rabbits by Moore and in patients suffering from acute lobar pneumonia by Moore and Chesney. These observers state that when patients receive by mouth 0.024 to 0.026 gram of optochin hydrochloride per twenty-four hours per kilogram of body weight, the serum acquires a pneumococcicidal action and that the drug is apparently helpful in the treatment of lobar pneumonia due to pneumococci.

The use of the drug in humans may give rise to amblyopia or amaurosis, which is generally transitory if the drug be discontinued.

when these symptoms appear. Moore and Chesney have collected from the literature 786 cases of lobar pneumonia treated with the drug, among which the mortality was 12.84 per cent.; the eye symptoms referred to above occurred in 4.4 per cent. of the cases and in one of these this disturbance was more or less permanent.

Tagendreich and Russo have shown that pneumococci subjected to sublethal concentrations of optochin in the test-tube can be rendered "fast" or resistant to the drug within a few days, and Morgenroth has shown that the same phenomenon can be observed to take place *in vivo* (mice) inefficiently treated. Moore and Chesney recovered a "fast" strain of pneumococcus from one of their fatal cases treated with optochin.

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## CHAPTER XVIII.

### MENINGOCOCCUS OR MICROCOCCUS (INTRACELLULARIS) MENINGITIDIS, AND THE RELATION OF IT AND OF OTHER BACTERIA TO MENINGITIS.

WHILE, as has already been stated, the pneumococcus is a cause of isolated cases of meningitis the meningococcus is the most frequent cause of purulent meningitis either sporadic or endemic. In 1887 Weichselbaum discovered a micrococcus in the exudate of cerebro-spinal meningitis in 6 cases, 2 of which were not complicated by pneumonia. He obtained it in pure cultures, studied its characteristics, and showed that this organism was clearly distinguishable from the pneumococcus and especially by its usual presence in the interior of pus cells, on which account he called it *Diplococcus intracellularis meningitidis*. In 1895 Jaeger and Scheurer drew especial attention to the etiological relationship of the organism to the epidemic form of cerebrospinal meningitis. They also believed it to be very probable that in most cases of primary meningitis it is from the mucous membrane of the nasal cavities and the sinuses opening out from them that both the diplococcus of pneumonia and the micrococcus intracellularis find their way through the blood or perhaps directly through the lymph channels to the meninges. The former we know to be almost constantly present in the nasal cavities, and the latter we have reason to believe is not infrequently there. The nasal secretion of carriers is more dangerous to the community than that of infected persons because carriers are not recognized as such usually and mingle freely with the general public. The prevalence of epidemics in winter and spring, a time favorable to influenza and pneumonia, also suggests the respiratory tract as the place of the infection and where an increase in virulence takes place. We do not as yet know why meningitis follows in some persons and not in others after infection of the mucous membranes.

**Morphology.**—This organism occurs as biscuit-shaped micrococci, usually united in pairs, but also in groups of four and in small masses, sometimes solitary, and small degenerated forms are found. It has no well-defined capsule. Cultures resemble strongly those of gonococci. In the sediment from spinal fluids (see Fig. 109) meningococci are both intra- and extracellular. When they are intracellular the prognosis is better, and frequently in cases where they are extracellular at first they later become intracellular.

**Staining.**—They stain with all the ordinary aniline dyes and are readily decolorized by Gram's solution. A smear from a culture shows characteristic irregularity in staining, some of the cocci taking the counter-

stain poorly and some staining deeply. The positive cocci described by Jaeger and others were probably contaminating organisms. The cells show no definite capsule.

**Cultivation.**—They grow between 25° and 38° C., best at about 35° C. They are most easily isolated on 2 per cent. glucose ascitic agar neutral

to phenolphthalein. They are kept well on semisolid media. The liver medium recommended by Dopter (p. 105) is an excellent one for stock transplants. The reaction of the media is very important. A culture can rarely be isolated on plain nutrient agar.

Different strains of meningococci vary somewhat in the ease with which they may be cultivated, their ability to ferment carbohydrates, virulence for animals, agglutinability, degree of digestibility in leukocytes, and power of resistance to immune serum.



FIG. 109.—*Diplococcus intracellularis meningitidis* in pus cells.  $\times 1100$  diameters.

After having been isolated for some time, a tolerably good growth develops at the end of forty-eight hours in the incubator. On semitransparent media (glucose-ascitic agar) the colonies may be seen as a flat layer, each about  $\frac{1}{4}$  inch in diameter, grayish white in color, finely granular, rather viscid, and non-confluent unless very close together. On Löffler's blood serum the growth forms round, whitish, shining, viscid-looking colonies, with smooth and sharply defined outlines; these may attain diameters of  $\frac{1}{16}$  to  $\frac{1}{8}$  inch in twenty-four hours. The colonies tend to become confluent and do not liquefy the serum. From the spinal fluid in acute cases, where the organisms are apt to be more abundant, a great many minute colonies may develop instead of a few larger ones. On agar plates the deep-lying colonies are almost invisible to the naked eye; somewhat magnified they appear finely granular, with a dentated border. On the surface they are larger, appearing as pale disks, almost transparent at the edges, but more compact toward the centres, which are yellowish gray in color. On blood agar or serum agar the growth is much more luxuriant than on plain agar and larger than the gonococcus. Cultivated in artificial media, while it often lives for weeks, it may die within four days, and requires, therefore, to be transplanted to fresh material at short intervals.

**Resistance.**—It is readily killed by heat, disinfectants, sunlight, and drying. A few cocci may remain alive for one to three days in the dried state. To maintain cultures it is necessary to make transfers frequently.

**Agglutination Characteristics.**—A considerable percentage of cultures of meningococci are relatively inagglutinable. Strains that are agglutinable respond to the agglutinins developed in an animal immunized with a true strain. Careful absorption tests are capable in many instances of separating true meningococci from other Gram-negative organisms. This serum reaction is practically never used for diagnosis

because it is so variable and unreliable. It may, however, be used in conjunction with other tests in standardizing protective serum.

Further work is necessary before we can know the limits of specific agglutinin-producing powers of the aberrant strains classed by Dopter (1909) under the term *parameningococcus*. Wollstein, testing many strains from different parts of the world, for agglutination, opsonization, complement-fixation and serum protection, comes to the conclusion that while certain strains exhibit differences enough to warrant the employment of them in the production of a polyvalent serum, they show too many variations in serum reactions to be separated into a distinct class. They should rather be considered special strains among meningococci. This conclusion is in general accord with those obtained through the extensive researches of Elser and Huntoon (1909).

The method used by us for making the agglutination test is as follows: A light emulsion is made by rubbing up the growth of an eighteen-hour neutral veal agar culture in salt solution. Equal amounts (0.5 c.c.) of an emulsion and the various serum dilutions are made, the tubes incubated at 55° C. overnight and the readings made in the morning.

**Pathogenesis.**—This organism has a low grade and very variable pathogenicity for laboratory animals. Following a suitable intraperitoneal injection the temperature of the guinea-pig falls, the hair stands out, the abdomen becomes distended and the muscles rigid. The animal hunches up in a corner and seems very sick. Not infrequently there is prolapse of the rectum. If the dose is fatal death usually occurs in from ten to forty-eight hours. At autopsy there is fluid exudate into the abdomen and sometimes into the pleural cavity, congestion or hemorrhage of the adrenals, hemorrhages into the mesentery, central tendon of the diaphragm and into the whole peritoneum. Frequently it is possible to recover the meningococcus from the heart's blood when live culture has been used. The organisms do not have to multiply to produce death and the autolysate or killed culture is just as fatal as live culture. Death is probably due to a bacterial poison freed by the disintegration of the meningococci. Very young cultures are apt to produce a septicemia. Rabbits injected either subcutaneously or intravenously lose weight rapidly.

**Pathogenicity for Man.**—The most marked lesions occur at the base of the brain. The cord is always affected. This is not true to the same extent in other bacterial infections. In some epidemics the course of the disease is very rapid. The mortality without serum treatment varies between 50 and 80 per cent.

**Presence of Meningococci in the Nasal Cavity of the Sick and Those in Contact with Them.**—In 1 of his 6 cases Weichselbaum succeeded in obtaining diplococci from the nasal secretion. In 1901 Albrecht and Ghon demonstrated them in healthy individuals. Scheurer, in 18 cases, found the diplococci in the nasal secretions during life. In 50 healthy individuals examined they were found in the nasal secretions of only 2, 1 being a man suffering at the time from a severe cold. This man, it is interesting to note, had been employed in a room which



had just previously been occupied by a patient with cerebrospinal meningitis. Lately, there has been a tendency to throw doubt on these findings, but from our experience in the 1906 epidemic in New York one can state that the meningococci are usually present in great numbers in the nose and nasopharynx in most cases of meningitis during the first twelve days of illness. After the fourteenth day they cannot usually be found. In 1 case Goodwin, of our laboratory, obtained them on the sixty-seventh day. She also found them in five persons out of sixty tested who had been in close contact with the sick, and in two of fifty medical students. We have found them since then in an interne convalescing from whooping-cough, who to his knowledge had not been in recent contact with any meningitis cases.

**Presence in Other Localities.**—In addition to the situation already noted the meningococcus is frequently found in the blood in the early days of the disease. Elser found it in blood cultures in 10 out of 40 cases. The meningococci have also been found in the herpes and in the urine, a fact to be considered in ordering the hygiene of the sick room.

**Complicating Infections.**—Occasionally we find secondary to the cerebrospinal meningitis, and due to the *Micrococcus pneumoniae*, cystitis, conjunctivitis, inflammation of the middle ear, arthritis, endocarditis, etc. The most frequent and serious complication of meningococcic meningitis is pneumonia probably due in many cases to the pneumococcus.

**Serum Treatment.**—It is difficult to apportion the credit for the production of the first protective serum. Bonhoff and Lepriere produced in animals a serum which showed definite protection. The world-wide epidemic beginning in 1904 stimulated a number of laboratories to produce sera in horses with the idea of treating human cases.

Thus Kolle and Wassermann, Jochmann, Flexner, and ourselves immunized horses. The usual method was to begin with cultures recently obtained from human cases and grow them on ascitic agar or plain nutrient agar in tubes. The growth was scraped off, added to physiological salt solution, and heated to 55° to 60° for one hour. Living cultures were often substituted later.

The original injections were quite small, being only one or two moderate-sized platinum loopfuls. Each succeeding injection was doubled in size each time, until the maximum dose of the growth on two Petri dishes was given, which size dose was continued to the end. The injections were given about every eight days. Horses give the best serum after eight months to one year. Kolle and Wassermann injected one horse with the watery extract of recent cultures. They used both the intravenous and subcutaneous methods.

**THE THERAPEUTIC USE OF SERUM.**—In 1905 there was inaugurated in Hartford the subcutaneous injections of diphtheria antitoxic serum by Dr. W. C. Wood. This influenced us to prepare and try the subcutaneous use of an antimeningococcus serum. The results in some 20 cases did not seem to establish the efficacy of the serum. No further serum was issued. Wassermann and Kolle, however, obtained somewhat favorable results

in a number of cases from the subcutaneous injection of a serum prepared by them. Meanwhile a serum prepared by Jochmann was employed by the intraspinal method in a series of cases. This method soon supplanted the subcutaneous injections.

The first successful use of an immune serum in cases of human cerebrospinal meningitis by the intraspinal method should therefore, so far as we know, be credited to Jochmann and the physicians who used his serum in the winter of 1905 and 1906. He reported a series of cases treated by the intraspinal method before the Congress for Internal Medicine held in Munich in April, 1906, and published his paper on May 17, 1906. He reported 40 cases, but gave details concerning only 17 patients, all treated by Kromer. Five of these patients died and twelve recovered, a mortality of 29 per cent. He directed that after lumbar puncture, 20 to 50 c.c. of fluid should be removed and then 20 c.c. of immune serum injected. These injections should be repeated once or twice if the fever did not abate or returned. He noticed in general a bettering of the headache, stiffness of neck, and mental condition. Jochmann showed that in animals colored fluids injected into the spinal canal in the lumbar region passed the full length of the canal. The serum was shown to possess both bactericidal and opsonic power.

Although the serum prepared in different laboratories in Europe was regularly used after Jochmann's report, it did not receive much attention in this country until Flexner, through his important experiments on infected monkeys, which demonstrated the value of the intraspinal injections of the serum, aroused medical interest and paved the way for him to try out the serum on a large scale. All cases treated by him were subjected to most careful bacterial tests and clinical observation. Eighteen months later, Flexner and Jobling published their report which fully corroborated the earlier results of Jochmann. The serum prepared at the Rockefeller Institute for Medical Research has been sent to many places, both in this country and in Europe. The results obtained have been of the utmost value in arriving at the value of the intraspinal treatment.

Details in regard to the administration of serum and of vaccine are given in Part III under Applied Therapy.

**Bacteriological Diagnosis.**—The fluid should be collected in a sterile container. It may be clear, cloudy, or bloody. If it is clear it may be a normal fluid or a fluid from a case of poliomyelitis or tuberculous meningitis.

If it is cloudy it may be due to the meningococcus, streptococcus pneumococcus, pneumococcus mucosus, influenza bacillus, or other rarer organisms. The blood in a bloody fluid may be due to a previous hemorrhage or to the accidental piercing of a vein. The two conditions may be differentiated by centrifuging the fluid. If the supernatant fluid is yellow or reddish the hemorrhage is old. Clear fluid indicates a recent hemorrhage. It is unfortunate to have much blood in the fluid for it obscures the microscopic picture and then unless the culture is positive it is difficult to make even a tentative diagnosis. Clear fluids

should always be centrifuged preferably for one hour at high speed. Cloudy fluids showing no organisms should also be centrifuged. The sediment should be used to make cultures and smears. The smears are examined: (1) for pus cells and (2) for tubercle bacilli if the fluid is clear or for other organisms if it is cloudy. The sediment from clear fluids should always be stained with the tubercle stain and that from cloudy fluids with Gram's stain. Once in a long while a cloudy fluid will be found to be tuberculous or a clear fluid to be due to some pyogenic organism but these occurrences are so rare as to be practically negligible. Gram's stain differentiates influenza bacillus, and meningococcus from the streptococcus, pneumococcus, and pneumococcus mucosus. The finding of Gram-negative cocci either intra- or extracellular is presumptive evidence of meningococcic meningitis, but it is always well to follow it up with a culture. In order that cultures may grow, fluids should be examined at the earliest moment possible, for meningococci in fluids over twelve hours old are frequently autolyzed so that they will not grow.

The following table gives the main differential points in making diagnosis from spinal fluids:

	Pressure.	Amount, c.c.	Appearance.	Cytology.	Bact.	Albumin.	Globulin.	Animal inoc.
Normal.	Normal.	5-10	Clear.	Very few cells.	Sterile.	±	—	Negative.
Meningismus.	Increased.	10-100	Clear.	Very few cells.	Sterile.	±	—	Negative.
Infantile paralysis.	Increased.	20-100	Clear; sometimes slight fibrin web.	Early polynucleosis; later lymphocytosis up to 95 per cent; endothelial cells.	Sterile.	+++	+++	Negative or pneumonia.
Tuberculous meningitis.	Increased.	30-120	Clear; fibrin web.	Lymphocytosis up to 95 per cent.	Tubercle bacilli.	+++++	+++++	Tuberculosis in 4 weeks.
Epidemi. cerebrospinal meningitis.	Increased.	5-120	Cloudy	Polynucleosis up to 98 per cent.	Meningococcus.	+++++	+++++	
Meningitis due to other organisms.	Increased.	20-100	Cloudy	Polynucleosis up to 98 per cent.	Infecting organisms.	+++++	+++++	

**Differential Diagnosis Distinguishing Meningococci from Gonococci.**—As a rule the portion of the body from which the organisms are obtained reveals their identity. When this is insufficient careful cultural and serological tests are required. McNeil has obtained a specific complement-fixation reaction (see p. 198).

**Other Gram-negative Cocci Resembling Meningococci.**—*Micrococcus Pharyngis* (Siccus) (von Lingelsheim), *Diplococcus Mucosus*, *Chromogenic Gram-negative Cocci*, *Micrococcus Catarrhalis*.—These may be differentiated by cultural characteristics.

**Other Organisms Exciting Meningitis.**—1. The *tubercle bacillus*. This is the most frequent cause of meningitis due to an organism other than the meningococcus.

2. The *Pneumococcus*. This diplococcus is one of the most frequent exciters of meningitis, both as a primary and a secondary infection.

3. The *Streptococcus pyogenes*, *Pneumococcus* and *Staphylococcus*. Meningitis due to these organisms is almost always secondary to some other infection, such as otitis, tonsillitis, erysipelas, endocarditis, suppurating wound of scalp and skull, etc.

4. The *Bacillus influenzae*. Numerous reports have been published of the presence of influenza bacilli in the meningeal exudate. Those that are reliable state in almost every instance that the meningitis is secondary to infection of the lungs, bronchi, and the nasal cavities with their accessory sinuses.

5. The *colon bacillus*, the *typhoid bacillus*, that of *bubonic plague* and of *glanders*, all may cause a complicating purulent meningitis.

6. In isolated cases of meningitis complicating otitis media and other infections, other bacteria, such as the *Micrococcus tetragenus*, the *Bacillus pyocyaneus*, the *gonococcus*, etc., may be found.

Meningitis due to other organisms than the meningococcus is almost invariably fatal.

### MICROCOCCUS CATARRHALIS (R. PFEIFFER).

Micrococci somewhat resembling meningococci are found in the mucous membranes of the respiratory tract. At times they excite catarrhal inflammation of the mucous membranes and pneumonia. These cocci are at present included under the designation of *Micrococcus catarrhalis*.

**Microscopic Appearance.**—They usually occur in pairs, sometimes in fours; never in chains. The cocci are coffee-bean in shape, slightly larger than the gonococcus, and are negative to Gram's stain.

The micrococci are not motile and produce no spores.

**Cultivation.**—They grow between 20° and 40° C., best at 37° C. and less rapidly at somewhat lower temperatures, developing on ordinary nutrient agar, as grayish white or yellowish white, circular colonies of the size of meningococci. The borders of the colonies are irregular and abrupt as though gouged out. They have a mortar-like consistency. On serum-agar media the growth is more luxuriant. Gelatin is not liquefied. Bouillon is clouded, often with the development of a pellicle. Milk is not coagulated, but dextrose serum media may be. Gas is not produced.

**Location of Organisms.**—In the secretion of normal mucous membranes they are occasionally present. In certain diseased conditions of the mucous membranes they may be abundant.

**Pathogenic Effects in Animals.**—For white mice, guinea-pigs, and rabbits, some cultures are as pathogenic as meningococci, while others are less so.

**Differential Points Distinguishing them from the Meningococci.**—These organisms have undoubtedly been at times confused. Some assert that the meningococci grow only above 25° C. Many cord cultures of meningococci grow below this point. Some assert that the meningococci will not grow on 5 per cent. glycerin agar. Many undoubted cultures do. Careful agglutinin-absorption tests are of great differential value, but can only be carried out safely by one accustomed to them. The meningococci tested by us have removed all the agglutinins acting upon meningococci from a specific meningococcus serum while the allied organisms have removed only about 60 per cent. of them. The probability is that the organisms described by different writers as *Micrococcus catarrhalis* were not all the same variety, and some of them were meningococci.

**Vaccine Therapy.**—Good reports have been made of the results of injecting the dead organisms in cases of infections due to this micrococcus.

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## CHAPTER XIX.

### THE GONOCOCCUS OR MICROCOCCUS GONORRHEÆ MICROCOCCUS MELITENSIS.

THE period at which gonorrhea began to inflict man is unknown. The earliest records make mention of it. Wherever civilized man has penetrated, gonorrhea is prevalent among the people. Except for a period after the fifteenth century it was generally recognized as a communicable disease and laws were made to control its spread. The differentiation between the lighter forms of gonorrhea and some other inflammations of the mucous membranes was, however, almost impossible until the discovery of the specific microorganism by Neisser in 1879.

The organism was first observed in gonorrheal discharges and described by him under the name of "gonococcus;" but though several attempted to discover a medium upon which it might be cultivated, it was reserved for Bumm, in 1885, to obtain it in pure culture upon coagulated human blood serum, and then after cultivating it for many generations to prove its infective virulence by inoculation into man. The researches of Neisser and Bumm established beyond doubt that this organism is the specific cause of gonorrhea in man. Gonorrhea is in almost all cases among adults transmitted through sexual intercourse. Gonorrheal ophthalmia is a frequent accidental infection at birth, and vaginitis in the young child is frequently produced by the carelessness of the nurse or mother carrying infection.

**Microscopic Appearance.**—Micrococci, occurring mostly in the form of diplococci. The bodies of the diplococci are broadened and, as shown in stained preparations, have an unstained division or interspace between two flattened surfaces facing one another, which give them their characteristic "coffee-bean" or "kidney" shape. The older cocci lengthen, then become constricted in their middle portion, and finally divide, making new pairs (Fig. 110). The diameter of an associated pair of cells varies according to their stage of development from  $0.8\mu$  to  $1.6\mu$  in the long diameter—average about  $1.25\mu$ —by  $0.6\mu$  to  $1\mu$  in the cross diameter.

**Extracellular and Intracellular Position of Gonococci.**—In gonorrhea, during the earliest stages before the discharge becomes purulent, the gonococci are found mostly free in the serum or plastered upon the epithelial cells, but later almost entirely in small, irregular groups in or upon the pus cells and epithelial cells, and always extranuclear. With the disappearance of the pus formation more free gonococci appear. Discharge expressed from the urethra usually contains more

free organisms than the natural flow. Gonococci sometimes appear irregular or granular, the so-called involution forms. These are found particularly in older cultures and in chronic urethritis of long standing. Single pus cells sometimes contain as many as one hundred gonococci and seem to be almost bursting and yet show but slight signs of injury. These diplococci are also found in or upon desquamated epithelial cells. There is still discussion as to whether the gonococci actively invade the pus cells or only are taken up by them. There is no evidence that the gonococci are destroyed by the pus cells (Fig. 111). In gonorrhea of the conjunctiva they are contained in the epithelial cells, sometimes in large numbers. They form dense groups which contain forms similar to those seen in older cultures, showing metachromatic granules in round, swollen, pale blue bodies. These groups finally present an appearance somewhat like the cell inclusions found in "trachoma" (p. 415).

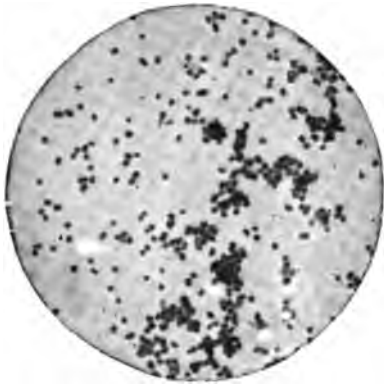


FIG. 110.—Smear from pure culture of gonococcus on agar.  $\times 1100$  diameters. (Heiman.)

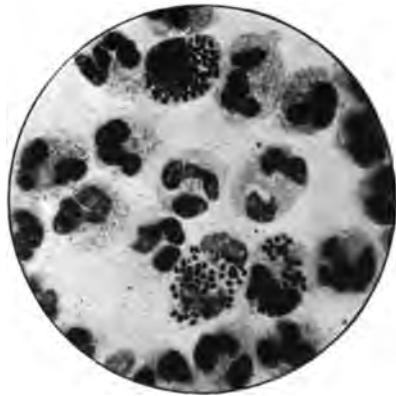


FIG. 111.—Gonococcus, fuchsin stain.  $\times 1000$  diameters. (Fränkel and Pfeiffer.)

**Staining.**—The gonococcus *stains* readily with the basic aniline colors (p. 75). Löffler's solution of methylene blue is one of the best staining agents for demonstrating its presence in pus, for, while staining the gonococci deeply, it leaves the cell protoplasm but faintly stained. Fuchsin is apt to overstain the cell substance. Beautiful double-stained preparations may be made from gonorrheal pus by treating cover-glass smears with methylene blue and eosin. Numerous methods for double staining have been employed, with the object of making a few gonococci more conspicuous. None of them has any specific characteristics such as the Gram stain. It is now established that gonococci from fresh cultures and from recent gonorrheal infections are, when properly treated by Gram's method, quickly and surely robbed of their color and take on the contrast stains. The removal of the stain from gonococci in old flakes and threads from chronic cases is not so certain. This difference is mostly due to the fact that

equally uniform specimens cannot be prepared. The decolorized gonococci are stained by dipping the films for a few seconds into a 1 to 10 dilution of carbol-fuchsin or a solution of Bismarck brown. This staining should be for as short a time as suffices to stain the decolorized organisms. This method of staining cannot be depended upon alone absolutely to distinguish the gonococcus from all other diplococci found in the urethra and vulvovaginal tract, for, especially in the female, other diplococci are occasionally found which are also not stained by Gram's method. It serves, however, to distinguish this micrococcus from the common pyogenic cocci, which retain their color when treated in the same way, and in the male urethra it is practically certain, as few organisms have been found in that location which in morphology and staining are identical with the gonococcus. It is certainly the most distinctive characteristic of the staining properties of the gonococcus, and it is a test that should never be neglected in differentiating this organism from others which are morphologically similar.

**Biology.**—Grows best at blood temperature; the limits being roughly 25° and 40° C. It is a facultative anaërobe. It is not motile and produces no spores.

**Culture Media.**—The gonococcus requires for its best growth the addition to nutrient agar of a small percentage of blood serum or some equivalent. The media which have proven of value may be found in the chapter on Media.

After continued cultivation gonococcus cultures frequently grow on media containing no serum. Occasional strains grow on ordinary glycerin or glucose nutrient agar and even on plain nutrient agar from the start.

**Viability.**—Cultures usually die in forty-eight to seventy-two hours when kept at room temperature. In the ice-box they may live for several weeks. They frequently live for one week in the thermostat at 36° C. on and in semisolid media.

**Appearance of Colonies.**—A delicate growth is characteristic. At the end of twenty-four hours there will have developed translucent, very finely granular colonies, with scalloped margin. The margin is sometimes scarcely to be differentiated from the culture medium. In color they are grayish white, with a tinge of yellow. The texture is finely granular at the periphery, presenting yellowish punctated spots of higher refraction in and around the centre (Fig. 112).

**Surface Streak Culture.**—Translucent grayish-white growth, with rather thick edges.

**Resistance.**—The gonococcus has but little resistant power toward outside influences. It is killed by weak disinfecting solutions and by desiccation in thin layers. In comparatively thick layers, however, as when gonorrheal pus is smeared on linen, it has lived for forty-nine days, and dried on glass for twenty-nine days (Heiman). It is killed at a temperature of 45° C. in six hours and of 60° in about thirty minutes.

**Occurrence of Gonococci in Nature.**—Outside of the human body or material carried from it gonococci have not been found.

**Pathogenesis.**—Non-transmissible to all animals. Both the living and dead gonococci contain toxic substances. Injected in considerable amounts into rabbits, they cause infiltration and often necrosis. Applied to the urethral mucous membrane there is produced an inflammation of short duration. In gonorrhea the secretion is believed to be due to these intracellular toxins. Repeated injections give only slight immunity. The filtrate of recent gonococcus cultures contains little toxin.

The etiological relation of the gonococcus to human gonorrhea has been demonstrated beyond question by the infection of a number of healthy men with the disease by the inoculation of pure cultures of the microorganism.

**Disease Conditions Excited by Gonococci.**—Affections due to this organism are usually restricted to the mucous membranes of the urethra, prostate, neck of bladder, cervix uteri, vagina, and conjunctiva. The conjunctival, vaginal, and rectal mucous membranes are much more sensitive in early childhood than in later life. The usual course of the inflammation is as follows: The gonococci first increase upon the mucous membranes which show congestion, infiltration with serous exudate and accumulation of leukocytes. The cocci then penetrate the epithelial layer down to the submucous connective tissue. Recovery or a prolonged chronic inflammation may then persist. The original infection of the urethra or vagina and cervix may remain localized or spread to adjacent parts or through blood and lymph be carried to all parts of the body. Gonococci thus cause many cases of endometritis, metritis, salpingitis, oöphoritis, peritonitis, proctitis, cystitis, epididymitis, and arthritis. Abscesses of considerable size, periostitis, and otitis are occasionally due to the gonococcus.

**Gonorrheal Ophthalmia.**—We have corroborated the statement of Stephenson and others that the gonococcus, though a frequent cause of ophthalmia neonatorum is not the only cause, in fact, in only about two-thirds of these cases is the gonococcus the cause of the inflammation.

**Endocarditis and Septicemia.**—Cases of gonococcus endocarditis and septicemia are not infrequent. Gonococcus septicemia may occur in connection with other localizations or alone. Nearly every year one or two of these cases are met with in every general hospital. In a considerable number of cases where gonococci are obtained from the blood the patients recover. The fever is sometimes typhoid-like in character.

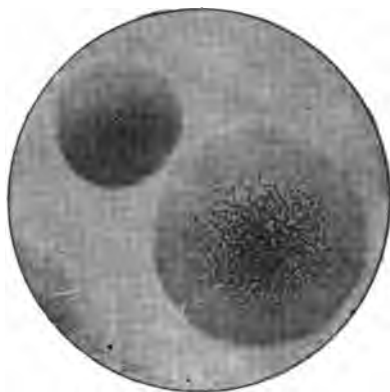


FIG. 112.—Colonies of gonococci on pleuritic fluid agar. (Heiman.)



**Complications.**—General infections with gonococci are often followed or accompanied by neuralgic affections, muscle atrophies, and neuritis. Urticaria occasionally occurs.

**Immunity.**—Immunity in man after recovery from infection seems to be only slight in amount and for a short period if present at all. It is known that the urethra in man or cervix uteri in woman may contain gonococci which lie dormant and may be innocuous in that person for years, but which may at any time excite an acute gonorrhea in another individual or, under stimulating conditions, in the one carrying the infection. Animals may, however, be immunized, and their blood is both bactericidal and slightly antitoxic.

**Therapeutic Use of Serum and Vaccine.**—The use of sera in acute gonorrheal joint inflammation has given in a considerable percentage of cases good results and seems to be worth trying. It seems to be useless in acute gonorrhea of the mucous membranes. Vaccines (heated cultures) have also been used with apparently real benefit in joint inflammations and even in very localized chronic infections of the urethra, bladder, and elsewhere. They have also been used in acute vaginitis in young children. In our cases the symptoms abated sooner than we expected, but the gonococci persisted. The dose is from twenty to a thousand millions given every three to seven days. The benefit of serum and vaccine in septicemia is doubtful. Sensitized serum seem to do good in some cases.

The use of vaccine and other points on the use of serum are given in Part III under Practical Applications of Vaccines and Serums.

**Complement-fixation.**—By the method of McNeil (p. 198) very good results have been obtained in the deep-seated chronic infections, but the reverse is true in the superficial acute cases. The method is used regularly in the New York City Health Department.

**Agglutination.**—Torrey has shown that gonococci resemble pneumococci in that there are a number of different strains which have different specific and but few common agglutinins. The agglutination test is of no practical value in diagnosis.

**Duration of Infections and of Contagious Period.**—There is no limit to the time during which a man or woman may remain infected with gonococci and infect others. We have had one man under observation in which twenty years had elapsed since exposure to infection, and yet the gonococci were still abundant. It is now well established that most of the inflammations of the female genital tract are due to gonococci, and that many of such infections are produced in innocent women by their husbands who are suffering from latent gonorrhea.

**Bacteriological Diagnosis of Gonorrhea.**—In view of the fact that occasional non-gonorrheal forms of urethritis exist, and also that micrococci morphologically similar to Neisser's diplococcus are sometimes found in the normal vulvovaginal tract of adults, it becomes a matter of importance to be able to detect gonococci when present, and to differentiate these from the non-specific organisms. Besides the diplococci which occur in old cultures and in chronic urethritis

of long standing sometimes take on a very diversified appearance. From a medicolegal and social stand-point, therefore, the differential diagnosis of the gonococcus has in certain cases a very practical significance.

There are three methods of differential diagnosis now available—the microscopic, the cultural, and complement-fixation. The method employed in the last test is given on p. 198. Animal inoculations are of no value, as animals are not susceptible, and, of course, human inoculations are usually impossible. In the microscopic diagnosis it should be borne in mind that after the acute serous stage has passed, the specific gonococci in *carefully made* preparations are always found largely within the pus cells. Diplococci morphologically similar to gonococci occurring in other portions of the field and outside of the pus cells should not be considered specific by this test only. It should also be remembered that the gonococci are decolorized by Gram's method, while other similar micrococci which occur in the urethra are, as a rule at least, not so decolorized. Organisms having these characteristics can for all practical purposes be considered as certainly gonococci if obtained from the urethra. From the vulvovaginal tract the certainty is not so great, since other diplococci are found in pus from this area more frequently than from the urethra which stain as gonococci; here cultures should also be made.

In *chronic urethritis* Heiman allows the patient to void his urine either immediately into two sterilized centrifuge tubes or first into two sterile bottles. The first tube will contain threads of the anterior urethra; the second tube will be likely to contain secretion from the posterior urethra and from the prostate gland if, while urinating, the patient's prostate be pressed upon with the finger. Tubes containing such urine are placed in the centrifuge and whirled for three minutes at twelve hundred or more revolutions per minute, at which speed the threads are thrown down. The centrifuged sediment will be found to contain most of the bacteria present, epithelial cells, and, at times, spermatozoa.

When the examinations are negative and it is important to be certain, either massage or injections of a solution of silver nitrate may be employed. The latter by causing a temporary irritation with increase of secretion will almost surely cause a discharge of gonococci if any infection is present.

In acute cases where the pus is abundant the specimen for examination may be collected, by passing a sterilized platinum-wire loop as far up into the urethra as possible and withdrawing some of the secretion.

**In vulvovaginitis** the procedure should be as follows:

For obtaining the vaginal material the labia are held well apart by an attendant wearing sterile rubber gloves. A sterile slender cotton swab is used which passes easily into the vagina without touching any external part but entrance. (If pus from the cervix is desired a speculum should be used.) The swab is rubbed gently about vaginal wall, then withdrawn and *rolled (not rubbed)* quickly

over a slide (slide sterilized and held face down if culture is to be made). *In making this smear care is used not to pass the swab over the same surface twice.* In this way a beautifully spread film is made. The swab is then returned to its holder (and covered and numbered if culture is to be made). The air-dried slide is rewrapped in its filter paper, numbered and sent to laboratory where it is stained by Gram's method (p. 78).

*The technic for making culture is as follows:* A small amount of rich sterile ascitic fluid is added to the tubes containing the swab. (If patient is at a distance from laboratory the ascitic fluid is sent in a separate tube, and is inoculated from swab just after smear is made; then swab is withdrawn from ascitic fluid tube, placed in its own tube, and both tubes are sent immediately to the laboratory.) After being stirred up in this fluid the swab is withdrawn and smeared in strokes radiating from the centre over ascitic agar (1-4) plates containing 2 per cent. glucose. Other plates of the same medium are stroked with platinum loopfuls of the ascitic fluid emulsion. From two to four plates are made and placed in thermostat at 36° C. After twenty-four hours the plates are examined and if gonococcus-like colonies are seen they are fished. Then a smear is made from the whole of one of the most characteristic streaks, stained by Gram's method and examined for gonococcus-like organisms.

*Grouping Cases.*—From the microscopic examination of *well-made* and *well-stained* smears (stained by Gram's method), and, when necessary, from cultures and from clinical appearance as well, the cases are divided into four groups, as follows:

1. *Positive cases, i. e.,* those showing leukocytes filled with morphologically typical gonococci in smear or showing typical cultures, or showing both.

2. *Suspicious cases, i. e.,* those showing in smears any suspicious intracellular diplococci and 50 per cent. or more of polymorphonuclear leukocytes.

3. *Observation cases, i. e.,* those showing in smears 50 per cent. or over of polymorphonuclear leukocytes, but no suspicious intracellular diplococci; or those having the clinical symptoms of discharge and inflammation and showing less than 50 per cent. of polymorphonuclear leukocytes.

4. *Negative cases, i. e.,* those showing in smears less than 50 per cent. polymorphonuclears, and no suspicious intracellular diplococci, and no clinical evidence of the disease.

*Isolation of Groups.*—Each group is kept isolated.

*Later Smears.*—From the first three groups smears are made once a week until a negative smear is obtained. From negative groups smears are made if any suspicious symptoms appear.

*Negative Diagnosis from Later Smears.*—Three successive *well-made* and *well-stained* negative smears from first three groups, at intervals of not longer than three days, are considered a *negative diagnosis*.

*Later Cultures.*—From cases where morphologically typical gonococci persist in smear, cultures may be made and gonococcus-like organisms isolated and studied to find out if they are true gonococci.

**Bacteria Resembling Gonococci.**—A few micrococci which resemble gonococci in form and staining have been described. These assume importance largely because they may be confused with the gonococcus. They occur occasionally on the conjunctival and vaginal mucous membranes. One of these microorganisms, the *Micrococcus catarrhalis* (see p. 283), has an importance of its own. Others are probably unimportant. When absolute certainty is demanded cultural and serological tests must be applied. Differential diagnosis from meningococci is given on p. 282.

### MALTA FEVER.

**The Micrococcus Melitensis.**—This microorganism was first discovered in the spleen in a case of Malta fever by Bruce in Malta in

1887. The disease is chiefly confined to the shores of the Mediterranean, but cases of it have been observed elsewhere. Infected goat-herds have been found in Texas.

**Clinical Symptoms.**—Prodromal symptoms follow an incubation period of five to fourteen days. Headache, sleeplessness, loss of appetite, or vomiting accompany a high fever. The fever lasts for weeks, with intermissions and remissions. A fever period of one to three weeks may occur from time to time during a period of many months. The spleen and liver are enlarged. The mortality is slight.

**Autopsy.**—The spleen is large and very soft. The liver is also large and congested. Both organs show parenchymatous degeneration.

**Distribution of Micrococci.**—These are most abundant in the blood and at the height of the fever and are present in organs and in the urine from the second day to the end of the disease.

**Morphology and Biology.**—Very small rounded or slightly oval organisms, about  $0.3\mu$  in their greatest diameter. They are usually single or in pairs. In old cultures involution, almost bacillary, forms occur. They are not motile and are Gram-negative.

**Cultivation.**—At  $37^{\circ}$  C. they grow rather feebly on nutrient agar and in broth. The colonies are not usually visible until the third day. They appear as small round disks, slightly raised, with a yellowish tint in the centre. The broth is slightly clouded after four to six days. The culture remains alive for several weeks or months. In gelatin the growth is very slow. Gelatin is not liquefied.

**Pathogenesis in Animals.**—Monkeys are susceptible. They pass through the disease much like man. They can be infected by subcutaneous or oral inoculation. Guinea-pigs and rabbits are less easily infected. Infected goats pass the organisms in feces, urine, and milk. The milk is believed to be the chief source of infection. By safeguarding the milk the disease has been largely eliminated. Contact infection cannot, however, be completely excluded. Horses and cows are also susceptible.

**Methods of Diagnosis.**—The diagnosis of Malta fever can frequently only be made with the help of bacteriological examination. Blood cultures during the febrile period or cultures of the urine are usually employed. An agglutination reaction with the patient's serum, in dilutions of 1 to 1000 or higher is diagnostic.

Animals injected with the coccus produce a serum agglutinating in high dilutions. This can be used to identify suspected cultures.

### MICROCoccus ZYMOGENS.

MacCallum and Hastings observed this micrococcus in a case of acute endocarditis. It has since been found in a few other pathological processes. It occurs in pairs and short chains. It grows well on agar, ferments lactose and glucose, and slowly liquefies gelatin.

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## CHAPTER XX.

### THE BACILLUS AND THE BACTERIOLOGY OF DIPHThERIA.

THE lesions of diphtheria are caused by toxemia. The concentrated poison at the seat of the exudate causes intense local inflammation, while in the more severe cases the absorbed poison diffused throughout the body causes widespread cellular injury, giving rise to definite lesions of the cells of muscle, nerve, and other tissues.

**Historical Notes.**—This specific contagious disease can be traced back under various names to almost the Homeric period of Grecian history. From time to time during the following centuries we hear of epidemics both in Italy and in other portions of the civilized world which indicate that the disease never absolutely ceased.

In 1771 Bard, an American, advanced strong reasons for believing that membranous croup and pharyngeal diphtheria were different manifestations of the same disease process.

In 1821 Bretonneau published his first essay on diphtheria in Paris and gave to the disease its present name. His observations were so extensive and so correct that little advance in knowledge took place until the causal relations of the diphtheria bacilli and their associated microorganisms to the disease began to be recognized.

**Evidence of Causal Relationship.**—As early as 1840 observers began to notice microorganisms in the pseudomembranes. Gradually the observations became more exact. The most importance was attributed to micrococci. In the year 1883, however, bacilli which were very peculiar and striking in appearance were shown by Klebs to be of constant occurrence in the pseudomembranes from the throats of those dying of true epidemic diphtheria. He described the peculiar staining of the organisms. One year later Löffler separated these bacilli from the other bacteria and grew them in pure culture. When he inoculated the bacilli upon the abraded mucous membrane of susceptible animals more or less characteristic pseudomembranes were produced, and frequently death or paralysis followed with characteristic lesions. These animal experiments have been fortified by a number of accidental human inoculations in laboratories with pure cultures of bacilli with subsequent development of diphtheria.

**The Diphtheria Bacillus.**—This bacillus is one of the most interesting of bacteria. Grown in the animal body or in suitable culture fluid, it produces a powerful toxin. Its morphology and staining are peculiar. Outside of the body it grows best on serum media.

**Morphology.**—When cover-glass preparations made from the exudate or from the cultures grown on blood serum, are examined, the diphtheria

bacilli are found to possess the following morphological characteristics: The diameter of the bacilli varies from  $0.3\mu$  to  $0.8\mu$  and the length from  $1\mu$  to  $6\mu$ . They occur singly and in pairs (see Figs. 113 to 120) and very infrequently in chains of three or four. The rods are straight or slightly curved, and usually are not uniformly cylindrical throughout their entire length, but are swollen at the end, or pointed at the ends and swollen in the middle portion. The average length of the bacilli in pure cultures from different sources frequently varies greatly, and even from the same culture individual bacilli differ much in their size and shape. This is especially true when the bacilli are grown in association with other bacteria. The two bacilli of a pair may lie with their long diameter in the same axis or at an obtuse or an acute angle. The bacilli possess no spores, but have in them highly refractive bodies, some of which are the starting-point for new bacilli. There are no flagella. For mode of division see p. 37.



FIG. 113.—One of the very characteristic forms of diphtheria bacilli from blood-serum cultures, showing clubbed ends and irregular stain.  $\times 1100$  diameters. Stain, methylene blue.



FIG. 114.—Extremely long form of diphtheria bacillus. This culture has grown on artificial media for over twenty years and produces great amounts of toxin.  $\times 1100$  diameters.

**Staining.**—The Klebs-Löffler bacilli *stain* readily with ordinary aniline dyes, and retain fairly well their color after staining by Gram's method. With Löffler's alkaline solution of methylene blue, and to a less extent with Roux's and dilute Ziehl's solutions (p. 78), the bacilli from blood-serum cultures especially, and from other media less constantly, stain in an irregular and extremely characteristic way. (See Fig. 113.) The bacilli do not stain uniformly. In many cultures round or oval bodies, situated at the ends or in the central portions, stain much more intensely than the rest of the bacillus, usually showing metachromatism (metachromatic granules. See p. 34 and Plate III.) Sometimes these highly stained bodies are thicker than the rest of the bacillus; again, they are thinner and surrounded by a more slightly stained portion. Other bacilli have barred staining. The bacilli stain in this peculiar manner at a certain period of their growth, so that only a portion of the organisms taken from a culture at any one time will show the characteristic staining. The young cultures have the most

regular forms, an eighteen-hour growth showing more clubbed forms than at twelve hours. After twenty-four hours the bacilli do not stain quite as well. In still older cultures it is often difficult to stain the bacilli,



FIG. 115.—Diphtheria bacilli characteristic in shapes, but showing even staining.  $\times 1000$  diameters. Stain, methylene blue.

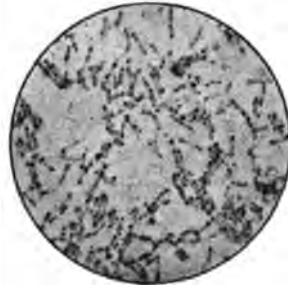


FIG. 116.—Non-virulent diphtheria bacilli, showing stain with Neisser's solutions. This appearance was formerly supposed to be characteristic of virulent bacilli. Bodies of bacilli in smear, yellowish brown; granules, dark blue.



FIG. 117.—*B. diphtheria* agar culture. Bacilli small and uniform in shape.  $\times 1000$  diameters.



FIG. 118.—*B. diphtheria*. Forty-eight hours' agar culture. Thick, Indian-clubbed rods and moderate number of segments. One year on artificial media.  $\times 1410$  diameters.



FIG. 119.—*B. diphtheria*. Forty-eight hours' agar culture. Many segments; long, Indian-clubbed ends. One year on artificial media.  $\times 1410$  diameters.



FIG. 120.—*B. diphtheria*. Twenty-four hours' agar culture. Coccus forms. Segmented granular forms on Löffler's serum. Only variety found; in cases of diphtheria at Children's Home.  $\times 1410$  diameters.

and the staining, when it does occur, is frequently not at all characteristic. The same round or oval bodies which take the methylene blue more intensely than the remainder of the bacillus are brought out still more distinctly by the Neisser stain (p. 78).

The Neisser stain has been advocated in order to separate the virulent from the non-virulent bacilli, without the delay of inoculating animals; but in our hands, with a very large experience, neither the Neisser stain nor other stains, such as the modifications of the Roux stain, have given much more information as to the virulence of the bacilli than the usual methylene-blue solution of Löffler. A few strains of virulent bacilli fail to show a marked characteristic stain, and quite a few pseudodiphtheria bacilli show the dark bodies. There are also in many throats bacilli which seem to have all the staining and cultural characteristics of the virulent bacilli, and yet have no relation to the disease diphtheria, that is, they produce no diphtheria toxin. As will be stated more fully later, nothing but animal inoculations with the suspected bacilli together with control injections of diphtheria antitoxin will separate harmless bacilli from those capable of producing diphtheria.

**The Morphology of the Diphtheria Bacillus on Serum-free Media.**—This varies considerably with different culture media employed. On glycerin agar or simple nutrient agar there are two distinct types. One grows as smaller and, as a rule, more regular forms than when grown on serum culture media (Fig. 117). The other type shows many thick, Indian-club forms with a moderate number of segments (Figs. 118–120). Short, spindle-, lancet-, or club-shaped forms, staining uniformly, are all observed. The bacilli which have developed in the pseudomembranes or exudate in cases of diphtheria resemble in shape young bacilli grown on agar.

**Biology.**—The Klebs-Löffler bacillus is non-motile and non-liquefying. It is *aërobic* and facultative *anaërobic*. It grows most readily in the presence of oxygen. It does not form spores. It begins to develop but grows slowly at a temperature of 20° C. or even less. It attains its maximum development at 37° C. In old cultures in fluid media Williams has observed fusion of one bacillus with another. The fused forms live the longest.

**Growth on Culture Media.**—**Blood serum.**—Blood serum, especially coagulated in the form of Löffler's mixture, is the most favorable medium for the growth of the diphtheria bacillus, and is used particularly for diagnostic purposes in examining cultures from the throats of persons suspected of having diphtheria. For its preparation see p. 101. If we examine the growth of diphtheria bacilli in pure culture on blood serum we shall find at the end of from eight to twelve hours small colonies of bacilli, which appear as pearl-gray, whitish-gray or, more rarely, yellowish-gray, slightly raised points. The colonies when separated from each other may increase in forty-eight hours so that the diameter may be one-eighth of an inch. The colonies lying together become confluent and fuse into one mass when the serum is moist. The diphtheria colonies after a growth of twelve hours become larger than those of the streptococci but remain smaller than those of the staphylococci.



**Growth on Agar.**—On 1 per cent. slightly alkaline, nutrient or glycerin-agar the growth of the diphtheria bacillus is less certain and luxuriant than upon blood serum; but the appearance of the colonies when examined under a low-power lens, though very variable, is often far more characteristic. (Fig. 121.) For this reason nutrient agar in Petri dishes is used to obtain diphtheria bacilli in pure culture. Certain strains of the diphtheria bacillus after having been transplanted for several generations on serum culture media, grow well, or fairly well, on suitable nutrient agar, but when fresh from pseudomembranes they grow on this medium with great difficulty, and the colonies develop

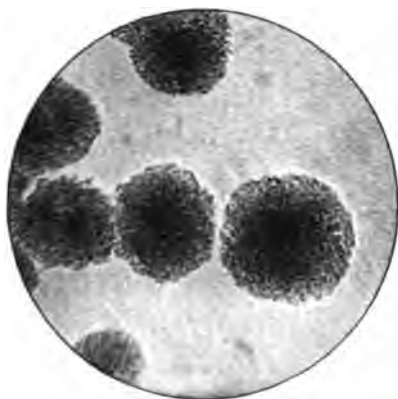


FIG. 121.—Colonies of diphtheria bacilli.  
× 200 diameters.

so slowly as to be covered up by the more luxuriant growth of other bacteria when present; or they may fail to develop at all.

If the colonies develop deep in the substance of the agar they are usually round or oval, and, as a rule, present no extensions; but if near the surface, commonly from one, but sometimes from both sides, they spread out an apron-like extension, which exceeds in surface area the rest of the colony. When colonies develop entirely on the surface they are more or less coarsely granular, and usually have a dark centre and vary markedly in

their thickness. The colonies from some are almost translucent; from others are thick and almost as luxuriant as the staphylococcus. The edges are sometimes jagged, and frequently shade off into a delicate lace-like fringe; at other times the margins are more even and the colonies are nearly circular.

Peculiarities in the growth of the diphtheria bacillus upon agar are of practical importance. If a large number of the bacilli from a recent culture are implanted upon a properly prepared agar plate a certain and fairly vigorous growth will always take place. If, however, the agar is inoculated with an exudate from the throat, which contains but a few bacilli, no growth whatever may occur, while the tubes of coagulated blood serum inoculated with the same exudate contain the bacilli abundantly. Because of the uncertainty, therefore, of obtaining a growth by the inoculation of agar with bacilli unaccustomed to this medium, agar is not a reliable medium for use in primary cultures for diagnostic purposes. A mixture composed of 2 parts of a 1.5 per cent. nutrient agar and 1 part of sterile ascitic fluid makes a medium upon which the bacillus grows much more luxuriantly, but not so characteristically.

**Isolation of the Diphtheria Bacillus from Plate Cultures.**—Nutrient or glycerin agar should be melted and poured in the Petri dish for this purpose. After it has hardened, the medium in a number of plates is streaked across with bacteria from colonies on the serum culture, which appear in size and color like the diphtheria bacilli. Other plates are made from a general mixture of all bacteria, selected, as a rule, from the drier portion of the serum. Still others are inoculated from the pellicles of ascitic broth cultures. The plates are left in

the incubator for about sixteen hours at 37° C. In the examination of the plates one should first seek for typical colonies, then, if these are not found, for any that look most nearly like the characteristic picture. Diphtheria colonies are very apt to be found at the edges of the streaks of bacterial growth. The fishings from the colonies are inoculated upon Löffler's blood serum, or into ascitic bouillon.

**Growth in Bouillon.**—The diphtheria bacilli from about one-half the cultures grow readily in broth slightly alkaline to litmus; the other strains grow feebly. The characteristic growth in neutral bouillon is one showing fine grains. These deposit along the sides and bottom of the tube, leaving the broth nearly clear. A few cultures in neutral bouillon and many in alkaline bouillon produce for twenty-four or forty-eight hours a more or less diffuse cloudiness, and frequently a film forms over the surface of the broth. On shaking the tube this film breaks up and slowly sinks to the bottom. This film is apt to develop during the growth of cultures which have long been cultivated in bouillon, and, indeed, after a time the entire development may appear on the surface in the form of a friable pellicle. The diphtheria bacillus in its growth, causes a fermentation of meat sugars and glucose, and thus, if these are present, changes the reaction of the bouillon, rendering it distinctly less alkaline within forty-eight hours, and then, after a variable time, when all the fermentable sugars have been decomposed, more alkaline again through the progressing fermentation of other substances. Among the products formed by its growth is the diphtheria toxin.

**Growth in Ascitic or Serum Bouillon.**—All varieties of diphtheria bacilli grow well in this medium, even when first removed from the throat. They almost always form a slight pellicle at the end of twenty-four to forty-eight hours. This culture medium is, as pointed out by Williams, of the greatest value in attempts to get pure cultures of the diphtheria bacillus from solidified serum cultures containing few bacilli among many other bacteria. Plate cultures are made from the pellicle. The fluid is prepared by adding to the nutrient bouillon 25 per cent. ascitic fluid or blood serum.

**Growth in Gelatin.**—The growth on this medium is much slower, more scanty, and less characteristic than that on the other media mentioned. This largely is on account of the lower temperature at which it must be used.

**Growth in Milk.**—The diphtheria bacillus grows readily in milk, beginning to develop at a comparatively low temperature (20° C.). The milk remains unchanged in appearance, as lactose is not fermented by the diphtheria bacillus.

**Pathogenesis.—In Lower Animals.**—The diphtheria bacillus through its toxins is, when injected into their bodies, pathogenic for guinea-pigs, rabbits, chickens, pigeons, small birds, and cats; also in a lesser degree for dogs, goats, cattle, and horses, but hardly at all for rats and mice. In spite of its pathogenic qualities for these animals true diphtheria occurs in them with extreme rarity. As a rule supposed diphtheritic inflammations in them are due to other bacteria which cannot produce disease in man. The cat is the only animal that we have known to contract true diphtheria from contact with the disease. Cobbett reports a case in a colt. At the autopsy of animals dying from the poisons produced by the bacilli, the characteristic lesions described by Löffler are found. At the seat of inoculation there is a grayish focus surrounded by an area of congestion; the subcutaneous tissues for

some distance around are edematous; the adjacent lymph nodes are swollen; and the serous cavities, especially the pleura and the pericardium, frequently contain an excess of fluid usually clear, but at times turbid; the lungs are usually congested, the suprarenals are markedly congested. In the organs are found numerous smaller and larger masses of necrotic cells, which are permeated by leukocytes. The heart and certain voluntary muscular fibers and nervous tissues usually show degenerative changes. Occasionally there is fatty degeneration of the liver and kidneys. The number of leukocytes in the blood is increased. From the area surrounding the point of inoculation virulent bacilli may be obtained, but in the internal organs they are only occasionally found, unless enormous numbers of bacilli have been injected. Paralysis, commencing usually in the posterior extremities and then gradually extending to the whole body and causing death by paralysis of the heart or respiration, is also produced in many cases in which the inoculated animals do not succumb to a too rapid intoxication. In a number of animals we have seen recovery take place three to six weeks after the onset of the paralysis.

**Tissue Changes in Natural (Human) Infection.**—The characteristic lesions are a pseudomembranous inflammation on some of the mucous membranes or occasionally on the surface of wounds and the general hyperplasias and parenchymatous inflammations produced by the absorbed toxic substances. Pneumonia is apt to occur as a complication of laryngeal diphtheria. The membrane may be simply a thin pellicle, which is easily removed without causing bleeding or it may be thick and firmly attached and leaving when removed a ragged bleeding surface. The tissue beneath the pseudomembrane is always intensely congested and often hemorrhagic. The cells show marked degenerative changes.

**Causes of Death.**—These are chiefly toxemia, laryngeal obstruction and bronchopneumonia. Septicemia due to other bacteria is frequently an additional factor.

**Diphtheria Toxin.**—This poison was assumed by Löffler (1884) to be produced by the bacilli, but it was first partially isolated by Roux and Yersin, who obtained it from cultures of the living bacilli by filtration through porous porcelain. It has not yet been successfully analyzed, so that its chemical composition is unknown, but it has many of the properties of protein substances, and can well be designated by the term active protein. It resembles in many ways the ferments. After injection into the body there is a latent period before its poisonous action appears. The poison produced is probably composed of a mixture of several nearly related toxins. Diphtheria toxin is totally destroyed by boiling for five minutes, and loses some 95 per cent. of its strength when exposed to 75° C. for the same time; 60° C. destroys very little. Lower temperatures only alter it very gradually. Kept cool and from light and air it deteriorates very slowly. Desiccated at low temperatures and kept dry in a vacuum it keeps unaltered for long periods. (See also pp. 151–153.)

**The Production of Toxin in Culture Media.**—The artificial production of toxin from cultures of the diphtheria bacillus has been found to depend upon definite conditions, which are of practical importance in obtaining toxin for the inoculation of horses, and also of theoretic interest in explaining why cases of apparently equal local severity have such different degrees of toxic absorption. The researches of Roux and Yersin laid the foundation of our knowledge. Their investigations have been continued by Theobald Smith, Spronck, ourselves, and others. After an extensive series of investigations we (Park and Williams) came to the following conclusions: Toxin is produced by fully virulent diphtheria bacilli at all times during their life when the conditions are favorable. Under less favorable conditions some bacilli are able to produce toxin while others are not. Diphtheria bacilli may find conditions suitable for luxuriant growth, but unsuitable for the production of toxin. The requisite conditions for good development of toxin, as judged by the behavior of a number of cultures, are a temperature from about 32° to 37° C., a suitable culture medium, such as a 2 per cent. peptone nutrient bouillon made from veal, of an alkalinity which should be about 9 c.c. of normal soda solution per liter above the neutral point to litmus, and prepared from a suitable peptone (Witte) and meat. The culture fluid should be in comparatively thin layers and in large-necked Erlenmeyer flasks, so as to allow of a free access of air. The greatest accumulation of toxin in bouillon is after a duration of growth of the culture of from five to nine days, according to the peculiarities of the culture employed. At a too early period toxin has not sufficiently accumulated; at a too late period it has begun to degenerate. In our experience the amount of muscle sugar present in the meat makes no appreciable difference in the toxin produced when a vigorously growing bacillus is used, so long as the bouillon has been made sufficiently alkaline to prevent the acid produced by the fermentation of the sugar from producing in the bouillon an acidity sufficient to inhibit the growth of the bacilli. With the meat as we obtain it in New York, we usually get better results with unfermented meat than with fermented. In Boston, with the same bacillus, Smith gets his best results from the bouillon in which the sugar has been fermented by the colon bacillus. Instead of colon bacilli, yeast may be added to the soaking meat, which is allowed to stand at about 25° C. The preliminary fermentation of the meat sometimes produces poisonous substances which are deleterious to the horses. We have obtained especially good results with veal broth made from calves two to four weeks old (bob veal). When strong toxin is desirable the muscle is separated from all fat, tendon and fibrous tissue before being chopped. (See p. 109.)

Under the best conditions we can devise toxin begins to be produced by bacilli from some cultures when freshly sown in bouillon some time during the first twenty-four hours; from other cultures, for reasons not well understood, not for from two to four days. In neutral bouillon the culture fluid frequently becomes slightly acid and toxin production may be delayed for from one to three weeks. The greatest accumulation of toxin is on the fourth day, on the average, after the rapid production of toxin has commenced. After that time the number of living bacilli rapidly diminishes in the culture, and the conditions for those remaining alive are not suitable for the rapid production of toxin. As the toxin is not stable at 35° C., the deterioration taking place in the toxin already produced is greater than the amount of new toxin still forming.

Bacilli, when repeatedly transplanted from bouillon to bouillon, gradually come to grow on the surface only. This characteristic keeps the bacilli in contact with the oxygen and seems to aid in the development of toxin.

**Comparative Virulence as Estimated by Toxin Production of Different Cultures.**—The virulence of diphtheria bacilli from different sources, as measured by their toxin production, varies considerably. Thus, as an extreme instance, 0.002 c.c. of a forty-hour bouillon culture of our most virulent strain will kill a guinea-pig, which would require

0.1 c.c. of culture of our least virulent strain to kill. This difference frequently depends on the unequal growth of the bacilli, one culture having fifty times as many bacilli as the other. When the different strains are grown on ascitic broth, upon which their growth is usually good, the majority of cultures are nearly equal in virulence, but some still show marked differences. Moreover, the diphtheria bacilli differ somewhat in the tenacity with which they retain their power to produce toxin when grown outside the body. The bacillus that we have used to produce toxin in the laboratory of the Board of Health has retained its power unaltered for twenty-two years in bouillon cultures. Other bacilli have apparently lessened their capacity for toxin production after being kept six months. Brown and Smith report on a culture in which 5 c.c. was the minimum toxic dose of the filtrate. The passage of diphtheria bacilli through the bodies of susceptible animals does not increase their toxic production to any considerable extent.

**Comparative Toxicity of Bacilli and Severity of Case.**—From the severity of an isolated case the toxicity of the bacilli cannot be determined. The presence of slight amounts of antitoxin in the blood of the person attacked and the association of other bacteria are at least two of the disturbing factors. The most toxic bacillus we have ever found was obtained from a mild case of diphtheria simulating tonsillitis. Another case, however, infected by this bacillus proved to be very severe. In localized epidemics the average severity of the cases probably indicates roughly the toxicity of the bacillus causing the infection, as here the individual susceptibility of the different persons infected would, in all likelihood, when taken together, be similar to that of other groups; but even in this instance special conditions of climate, food, or race may influence certain localities. It must be remembered that bacilli of like toxic power may differ in their liability to infect the mucous membrane; that is, the virulence of the organism may not go hand in hand with the toxicity.

**Toxic Bacilli in Healthy Throats.**—Fully toxic bacilli have frequently been found in healthy throats of persons who have been brought in direct contact with diphtheria patients or diphtheria carriers without contracting the disease. It is therefore apparent that infection in diphtheria, as in other infectious diseases, requires not only the presence of toxic bacilli, but also a susceptibility to the disease, which may be local or general. We now know that 70 per cent. of all persons are protected from infection because of having antitoxin present in their blood. Among the predisposing influences which contribute to the production of diphtheritic infection may be mentioned the breathing of foul air and living in overcrowded and ill-ventilated rooms, impure food, certain diseases, more particularly catarrhal inflammations of the mucous membranes, and depressing conditions generally. Under these conditions an infected mucous membrane may become susceptible to disease. In connection with Beebe (1894) we made an examination of the throats of 330 healthy persons who had not come in contact, so far as known, with diphtheria, and we found

toxic bacilli in 8, only 2 of whom later developed the disease. In 24 of the 330 healthy throats non-toxic bacilli similar to the toxic diphtheria bacillus were found. Very similar observations have since been made in Boston and by others in many widely separated countries. In 1905 Von Sholly in our laboratory examined 1000 throats of those who had not knowingly been in contact with diphtheria and found toxic diphtheria bacilli in 0.5 per cent. of the cases. We have found toxic bacilli in about 5 per cent. of cases of scarlet fever. This indicates they are more prevalent in throats than a single culture from normal cases indicate.

**Persistence of Diphtheria Bacilli in the Throat.**—The continued presence of toxic diphtheria bacilli in the throats of patients who have recovered from the disease has been demonstrated by all investigators. In the investigations of 1894 we found that in 304 of 605 consecutive cases the bacilli disappeared within three days after the disappearance of the pseudomembrane; in 176 cases they persisted for seven days, in 64 cases for twelve days, in 36 cases for fifteen days, in 12 cases for three weeks, in 4 cases for four weeks, and in 2 cases for nine weeks. Since then we have met with a case in which they persisted with full toxicity for eight months. It is safe to say that in over 10 per cent. of the cases a few bacilli persist two weeks after the disappearance of the exudate and in over 1 per cent. four weeks. It is extremely difficult to prove that a case is absolutely clear, as the bacilli may remain hidden in the epithelial cells of some tonsillar crypt and not be detected by cultures.

**Diphtheria-like Bacilli Not Producing Diphtheria Toxin.**—In the tests of the bacilli obtained from hundreds of cases of suspected diphtheria which have been carried out during the past twenty years in the laboratories of the Health Department of New York City, in over 95 per cent. of cases the bacilli derived from exudates or pseudomembranes and possessing the characteristics of the Löffler bacillus have been found to be toxic, that is, producers of diphtheria toxin. But there are, however, in inflamed throats as well as in healthy throats, either alone or associated with the toxic bacilli, occasionally bacilli which, though morphologically and in their behavior on culture media identical with the Klebs-Löffler bacillus, are yet producers, at least in artificial culture media and the usual test animals, of no diphtheria toxin. Between bacilli which produce a great deal of toxin and those which produce none we find a few minor grades of toxicity. We believe, therefore, in accordance with Roux and Yersin these non-toxic bacilli should be considered as possible attenuated varieties of the diphtheria bacillus which have lost their power to produce diphtheria toxin. This supposition is, however, not proven and it may be that the ancestors of these bacilli were never toxin producers. These observers, and others following them, have claimed that the toxic bacilli can be artificially attenuated; but the reverse has not been proven that bacilli which produce no specific toxin have later been found to develop it. In our experience some cultures hold their toxicity even

when grown at 41° C. for a number of months, while others become slightly attenuated rather quickly. We have never yet been able to change a toxic culture into an absolutely non-toxic one, and we have never accomplished the reversed process. We believe, therefore, that for practical purposes bacilli which produce no toxin in animals can be disregarded as a possible source of human diphtheria.

Among the non-toxic diphtheria-like bacilli which are obtained frequently from normal or slightly inflamed throats or from other mucous membranes are some that may be slightly virulent for guinea-pigs, since they may kill, as we found, in doses of 2 to 5 c.c. of broth culture subcutaneously or intraperitoneally injected. Animals are not protected by diphtheria antitoxin from the action of these bacilli, showing that their poisonous action is not due to diphtheria toxin. At autopsy the bacilli are usually found more or less abundantly in the blood and internal organs. The fact that large injections of antitoxin serum hastens the death of guinea-pigs injected with these bacilli has given rise to the notion that injections of antitoxin might be dangerous in persons in whose throats these bacilli were present, either as saprophytes, or possibly, as inciters of slight disease. It is not the antitoxin, but the serum, which in large doses injures the vitality of the guinea-pigs and so slightly hastens death. The amount of serum required to produce this effect is far in excess of that properly given in man. These bacilli were first described by Davis from our laboratory and later by Hamilton in 1904. The possibility of their being present affords no reason to avoid giving antitoxin in suspected cases. When pathogenic in man they are usually only feebly so.

**Diphtheroids.**—There are a large number of small more or less irregular bacilli bearing some morphological resemblance to the diphtheria bacillus which have been loosely termed diphtheroids. They have been found in the nose, throat, eye and other parts of the body, both in health and in disease (Hodgkin's disease, leukemia, etc.). Several of them, *e. g.*, *B. hoffmanni*, *B. xerosis*, are considered distinct species (see below). Since this type of organism is so widely distributed, any specific pathogenic properties attributed to them, unless positive proof is offered, must be received with reserve.



FIG. 122.—Pseudodiphtheria bacilli. (*B. hoffmanni*.)

**Bacillus Hoffmanni (Pseudodiphtheria Bacilli).**—These bacilli are rather short, plump, and more uniform in size and shape than the true Löffler bacillus (Fig. 122). On blood serum their colony growth is very similar to that of the diphtheria bacilli. The

great majority of them in any young culture show no polar granules when stained by the Neisser method, and stain evenly throughout with the alkaline methylene blue solution. They do not produce acid by the fermentation of glucose, as do all known virulent and many non-

virulent diphtheria bacilli; therefore there is no increase in acidity in the bouillon in which they are grown during the first twenty-four hours from the fermentation of the meat sugar regularly present. They are found in varying abundance in different localities in New York City, in about 1 per cent. of the normal throat and nasal secretions, and seem to have now at least no connection with diphtheria; whether they were originally derived from diphtheria bacillus is doubtful. They have been called *pseudodiphtheria bacilli*, and more properly *B. hofmanni*. In bouillon they grow, as a rule, less luxuriantly than the diphtheria bacilli. Some of the varieties of the pseudodiphtheria bacilli are as long as the shorter forms of the virulent bacilli. When these are found in cultures from cases of suspected diphtheria they may lead to an incorrect diagnosis. These bacilli are found occasionally in all countries where search has been made for them. There are also some varieties of diphtheroids which resemble the short *B. hofmanni* in form and staining, but which produce acid in glucose bouillon.

**Bacillus Xerosis.**—Diphtheria-like bacilli were found by Kutschert and Neisser (1884) in the condition known as xerosis conjunctivæ. Since then they have been found frequently in normal conjunctivæ. These bacilli went by the name of xerosis bacilli. Under this name, no doubt, different observers have placed several varieties of bacilli morphologically somewhat similar to the diphtheria bacilli. Knapp first limited the name to bacilli that ferment saccharose and not dextrin, in contradistinction to diphtheria bacilli which ferment dextrin and not saccharose. The fermentation tests for three of the diphtheria group, according to Knapp, may be shown as follows:

Species.	Dextrose.	Levulose.	Galactose.	Maltose.	Saccharose.	Dextrin.
<i>B. diphtheria</i> . . .	+	+	+	+	—	+
<i>B. xerosis</i> . . .	+	+	+	+	+	—
<i>B. hofmanni</i> . . .	—	—	—	—	—	—

NOTE.—One per cent. sugar in Hiss serum-water media.

These reactions do not separate a large number of the non-virulent bacilli morphologically like the diphtheria bacilli mentioned before.

**Micro-aërophilic "Diphtheroids."**—Certain "diphtheroid" bacilli chose for their most abundant growth that part of the medium which contains only a small amount of oxygen. Thus in a deep shake agar tube culture, a band of growth appears a short distance from the surface of the medium and few colonies appear below this band. Among these microaërophilic bacilli (see p. 120) the acne bacillus is of interest to us because of its probable etiological importance in acne vulgaris.

**Bacillus Acne.**—A short, rather plump, irregularly shaped, bacillus was first reported as occurring in acne pustules and comedones by Unna in 1894. A morphologically similar bacillus was grown by Sabouraud, in 1897, in pure cultures on an acid glycerin agar. Whether or not this was the acne bacillus remains doubtful, since pure aërobic cultures are so difficult to obtain. In 1907 Halle and Civatte found an anaërobe in the sebaceous follicles from all faces examined. This threw doubt



on the etiological relationship of this bacillus to acne. But Gilchrist and others found a similar bacillus most abundantly in acne lesions, though it was frequently accompanied by a small coccus. Flemming, in 1909, found a similar anaërobic diphtheroid in large numbers in acne vulgaris and obtained abundant anaërobic growths on oleic acid glycerin agar.

The bacillus now accepted as *B. acne* has the following characteristics: The cultures are made up of short, rather irregularly club-shaped, non-motile rods averaging  $2\mu$  by  $0.5\mu$ . These are Gram-positive and take ordinary stains well. They grow best with only a small amount of oxygen; that is, in shake tube cultures the most abundant growth appears in the form of a hazy band about half an inch from the surface of the medium and about a fourth of an inch thick. This develops in about three to five days at  $36^{\circ}\text{C}$ .

In our laboratory a semisolid medium (p. 99) has been found to be best for the isolation of this bacillus. A shake culture is made in a tube of this medium. If the tube, after sterilization by carbolic acid, is cut just below the zone of growth and new shake culture tubes made from a portion taken from the periphery of this zone, repeating on subcultures if necessary, a pure culture will finally result.

The question of the efficacy of vaccine treatment in acne vulgaris with *B. acne* and with *Staphylococcus pyogenes* is taken up in the chapter on the Therapeutic Application of Vaccines in Section III.

**Persistence of Varieties of the Bacillus Diphtheria and of Diphtheria-like Bacilli.** The fact that there are distinct differences between strains of bacilli producing diphtheria toxin which are as great as between these and some strains of bacilli producing no diphtheria toxin has, we think, been fully established.

But that such varieties are true subspecies with constant characteristics, one variety not changing into another of the established forms, has not been accepted by all. On the contrary, the opinion is held by some investigators that all of the various forms of diphtheria-like bacilli are the result of more or less transitory variations of the same species and hence that the toxic forms are the result of a rapid adaptation to environment and consequent pathogenesis of the non-toxic forms, both typical and atypical. This question is of great practical importance in methods of handling the persons harboring these bacilli.

Wesbrook, Wilson, and McDaniel, make a provisional classification based upon the morphology of the individual bacilli, into three groups, called granular, barred, and solid, two of the groups into seven types and the other into five, two of the types corresponding with those in the other groups not having been seen. They state that there is generally a sequence of types in the variations which appear throughout the course of the disease, the granular types, as a rule, predominating at the outset of the disease, and these giving place wholly or in part to the barred and solid types shortly before the disappearance of diphtheria-like organisms.

The inference drawn from this work is that the diphtheria bacillus may be rather easily, especially in the throat, converted into non-granular, solidly staining forms of the "pseudodiphtheria" type, and that the converse may occur, and that therefore all diphtheria-like bacilli must be considered a possible source of danger.

In studying the subject Williams (1902) came to the following conclusions: Though some cultures change on some of the media, each changes in its own way, and each culture still has its distinct individuality. After many culture generations, especially when transplanted at short intervals, the different varie-

ties of toxic diphtheria bacilli tend to run in lines parallel with a common norm, which seems to be a medium-sized, non-segmented bacillus, producing granules in early cultures on serum and growing well on all of the ordinary culture media. The non-toxic morphologically typical bacilli must be classed with the toxic varieties as one species, though there is little doubt that more minute study would show that the former constitute a distinct group. The atypical pseudo forms, however, which show no tendency to approach the norm of the typical forms, must be classed as distinct species. All of the pseudo and the non-toxic morphologically typical varieties when inoculated into the peritoneum of guinea-pigs in immense doses cause death. In studying successive direct smears from the throats of diphtheria patients no evidence of change from one type to the other was noted. Attempts have been made to give more virulence and some toxicity to a few of these varieties by successive peritoneal inoculations, and by growing the organisms in symbiosis with several other organisms, but in no instance has any increase of pathogenicity or decided change in morphological or cultural characteristics been noted.

Since there are so many different forms or varieties of diphtheria-like bacilli, it is quite possible that some of them are derived from strains of the diphtheria bacillus and that under certain conditions they readily regain its characteristics. This seems to be the only way to explain the apparent discrepancies in the results obtained by different observers. Such closely related varieties, however, do not appear to be common and we have up to the present been unable to obtain them. So we may safely say that in this region, at least, non-toxic diphtheria-like organisms retain their characteristics under various artificial and natural conditions, and that they may be regarded from a public health stand-point as harmless.

**Resistance to Heat, Drying, and Chemicals.**—The thermal death-point with ten minutes' exposure is about 60° C., with five minutes 70° C. Boiling kills almost instantly. The bacillus has about the average resistance of non-spore-bearing bacteria to disinfectants. In the dry state and exposed to diffuse light diphtheria bacilli usually die in from a few hours to a few days, but they may live for months; when in the dark, or protected by a film of mucus or albumin, they may live for even longer periods. Thus we found scrapings from a dry bit of membrane to contain vigorous and toxic living bacilli for a period of four months after removal from the throat, and if the membrane had not been at that time completely used, living bacilli could probably have been obtained for a much longer period. On slate- and lead-pencils, toys, tumblers, as well as on paper money, they may live for several weeks, while on metal coins they die in twelve to thirty-six hours. In culture media, when kept at the blood heat, they usually die after a few weeks; but under certain conditions, as when sealed in tubes and protected from heat and light, they retain their life and toxicity for years. The bacillus is not sensitive to cold, for we found about 10 per cent. of the bacilli to retain their vitality and toxicity after exposure for two hours to several hundred degrees below zero. At temperatures just below freezing they may remain alive for a number of weeks.

**Transmission of Diphtheria.**—The possibility of the transmission of diphtheria from animals to man cannot be disputed; we have met

two instances in which cats had malignant diphtheria, and many other animals can be infected, but as we have said there are few authentic cases of such transmission on record. So-called diphtheritic disease in animals and birds is usually, if not always, due to other microorganisms than the diphtheria bacilli.

The toxic bacilli have been found on soiled bedding or clothing of a diphtheria patient, or drinking-cups, candy, shoes, hair, slate-pencils, etc. These sources of infection by which the disease may be indirectly transmitted are, however, the less important ones. The usual source of the bacilli are the discharges of diphtheria patients; the secretions from the nose and throat of convalescent cases of diphtheria in which the toxic bacilli persist, and from the healthy throats of individuals who acquired the bacilli from being in contact with others having virulent germs. When we consider the number of healthy carriers and that it is only the severe types of diphtheria that remain isolated during their actual illness, the wonder is not that so many, but that so few, persons contract the disease.

**Susceptibility to and Immunity against Diphtheria.**—An individual susceptibility, both general and local, to diphtheria, as in all infectious diseases, is necessary to contract the disease. Age has long been recognized to be an important factor in diphtheria. Children within the first six months of life are but little susceptible, exceptionally infants of a few weeks are attacked. The time of greatest susceptibility is between the second and tenth year. After the tenth year susceptibility decreases. Young animals born of mothers immune to diphtheria possess nearly the same degree of immunity as their mothers. They gradually lose this but retain traces up to six to twelve months. The human infant is now known to receive immunity from its mother. This immunity has been shown to depend on the presence of antitoxin.

**The Persistence in Man's Blood of Homologous and Alien Antitoxin.**—Antitoxins and other antibodies produced in an animal disappear more rapidly when introduced into the blood of another species than into one of the same species. In man an alien serum must be used in all except exceptional cases.

In our experiments in guinea-pigs we have found that the homologous antitoxin was retained in appreciable amounts for at least six months, while the heterologous antibodies were noticeable to the same extent for only four weeks. There is a rather rapid loss of horse-produced antitoxin during the first few days and then a slow loss, becoming more and more gradual until final elimination at the end of ten days to three weeks. The larger the amount of antibodies injected the longer will be the time before the elimination of effective amount.

For a discussion of the nature of antitoxin see page 168. For a presentation of the therapeutic use of antitoxin see Part III.

**Mixed Infection in Diphtheria.**—Toxic diphtheria bacilli are not the only bacteria present in human diphtheria. Various cocci and bacilli more particularly streptococci, staphylococci, pneumococci, and influ-

enza bacilli, are always found actively associated with Löffler's bacillus in diphtheria, playing an important part in the disease and leading often to serious complications (sepsis and bronchopneumonia). Investigations indicate that when other pathogenic bacteria are associated with the diphtheria bacilli they mutually assist one another in their attacks upon the mucous membrane, the streptococcus being particularly active in this respect, often opening the way for the invasion of the Löffler bacillus into the deeper tissues or supplying needed conditions for the development of its toxin. In most fatal cases of bronchopneumonia following laryngeal diphtheria we find not only abundant pneumococci or streptococci in the inflamed lung areas, but also in the blood and tissues of the organs. As these septic infections due to the pyogenic cocci are in no way influenced by the diphtheria antitoxin, they frequently are the cause of the fatal termination. Other bacteria cause putrefactive changes in the exudate, producing alterations in color, *e. g.*, *B. pyocyaneus* and offensive odors, *e. g.*, *B. fusiformis*.



FIG. 123.—Vincent's bacillus with accompanying spirochetes.

**Pseudomembranous Exudative Inflammations Due to Bacteria other than the Diphtheria Bacilli.**—The diphtheria bacillus, though the most usual, is not the only microorganism that is capable of producing pseudomembranous inflammations. There are numerous bacteria present almost constantly in the throat secretions, which, under certain conditions, can cause local lesions very similar to those in the less-marked cases of true diphtheria. The streptococcus and pneumococcus are the two forms most frequently found in these cases, but there are also others, such as Vincent's bacillus, which, under suitable conditions, excite this form of inflammation, but without serious constitutional symptoms.

The pseudomembranous angina accompanying scarlet fever, and to a less extent other diseases, may not show the presence of diphtheria

bacilli, but only the pyogenic cocci, especially streptococci, or, more rarely, some varieties of little-known bacilli. The deposit covering the inflamed tissues in these non-specific cases is, it is true, usually but not always, rather an exudate than a true pseudomembrane.

**Relation of Bacteriology to Diagnosis.**—We believe that all experienced clinicians will agree that, when left to judge solely by the appearance and symptoms of a case, there are certain mild exudative inflammations of the throat of similar appearance some of which transmit diphtheria while others do not.

The doubtful cases that have the diphtheria bacilli in the exudate, are capable of giving true characteristic diphtheria to others, or later develop it characteristically themselves, while those in whose throats no diphtheria bacilli exist can under no condition give true characteristic diphtheria to others or develop it themselves. It is, indeed, true, as a rule, that cases presenting the appearance of ordinary follicular tonsillitis in adults are not due to the diphtheria bacillus. On the other hand, in small children mild diphtheria very frequently occurs with the semblance of rather severe ordinary follicular tonsillitis, due to the pyogenic cocci; and in large cities where diphtheria is prevalent all such cases must be watched as being more or less suspicious. Most observers agree with us in thinking that if in any case exposure to diphtheria is known to have occurred, even a slightly suspicious sore throat should be regarded as probably due to the diphtheria bacilli. If, on the other hand, no cases of diphtheria have been known to exist in the neighborhood, even cases of a more suspicious nature would probably not be regarded as diphtheria. Now that we know about 50 per cent. of children have a fairly constant supply of antitoxin in their blood, we have reason to believe that many of these doubtful cases are simply diphtheria carriers in which the bacilli are taking little or no part in making the lesions. Like any carriers they are dangerous to others.

The presence of irregular-shaped patches of adherent grayish or yellowish-gray pseudomembrane on some other portions than the tonsils is, as a rule, an indication of the activity of the diphtheria bacilli. Restricted to the tonsils alone, their presence is less certain.

Occasionally, in scarlatinal angina or in severe phlegmonous sore throats, patches of exudate may appear on the uvula or borders of the faucial pillars, and still the case may not be due to the diphtheria bacilli; these are, however, exceptional. Thick, grayish pseudomembranes which cover large portions of the tonsils, soft palate, and nostrils are almost invariably the lesions produced by diphtheria bacilli.

The very great majority of cases of pseudomembranes of exudative laryngitis, in the coast cities at least, whether an exudate is present in the pharynx or not, are due to the diphtheria bacilli. Nearly all membranous affections of the nose are true diphtheria. When the membrane is limited to the nose the symptoms are, as a rule, very slight; but when the nasopharynx is involved the symptoms are usually grave.

Most cases of pseudomembranes and exudates, entirely confined

to portions of the tonsils in adults, are not due to the diphtheria bacilli. Cases presenting the appearances found in scarlet fever, in which a thin grayish membrane lines the borders of the uvula and faucial pillars, are rarely diphtheritic. As a rule pseudomembranous inflammations complicating scarlet fever, syphilis, and other infectious diseases are due to the activity of the pathogenic cocci and other bacteria, induced by the inflamed conditions of the mucous membranes due to the scarlatinal or other poison. The possibility of these persons being carriers of diphtheria bacilli must always be kept in mind.

Paralysis following a pseudomembranous inflammation is an almost positive indication that the case was one of diphtheria, although slight paralysis has followed in a very few cases in which careful cultures have revealed no diphtheria bacilli. These, if not true diphtheria, must be considered very exceptional cases.

**Bacteriological Diagnosis.**—From the above it is apparent that fully developed characteristic cases of diphtheria are readily diagnosticated, but that many of the less marked, or at an early period undeveloped, cases are difficult to differentiate. In these cases cultures are of the utmost value, since they enable us to isolate those in which diphtheria-like bacilli are found, and to give preventive injections of antitoxin to both the sick and those in contact with them, if this has not already been done. As a rule cultures do not give us as much information as to the gravity of the case as the clinical appearances, for before the lapse of the twelve hours required for the laboratory report, the extent of the disease usually allows a diagnosis. The reported absence of bacilli in a culture must be given weight in proportion to the skill with which the culture was made, the suitability of the media, the location of the disease, and the knowledge and experience of the one who examined it.

Diphtheria does not occur without the presence of the diphtheria bacilli; but there have been many cases of diphtheria in which, for one or another reason, no bacilli were found in the cultures by the examiner. In many of these cases later cultures revealed them. The reverse is also true, the presence of diphtheria bacilli in throats without clear clinical signs of diphtheria in no sense makes it a case of diphtheria. In a convalescent case the absence of bacilli in any one culture indicates that there are certainly not many bacilli left in the throat, but even repeated cultures cannot absolutely prove their total absence, for in some deep tonsillar crypt a few bacilli may remain in the epithelial cells. The physician must have intelligence to use advantageously laboratory findings.

**Technic of the Bacteriological Diagnosis.**—*Collection of the Animal Blood Serum and its Preparation for Use in Cultures.* (See p. 101.)

**Swab for Inoculating Culture Tubes.**—The swab we prefer to use to inoculate the serum is made as follows: A stiff, thin, iron rod, 6 inches in length, is roughened at one end by a few blows of a hammer, and about this end a little absorbent cotton is firmly wound. Each swab is then placed in a separate glass tube, and the mouths of the tubes are plugged with cotton. The tubes and rods are then sterilized by dry heat at about 150° C. for one hour, and stored for future use. These iron rods have proved more serviceable for making

inoculations than platinum-wire needles or wooden sticks, especially in young children and in laryngeal cases. It is easier to use the cotton swab in such cases and it gathers up so much more material for the inoculation that it has seemed more reliable. The wood unless very carefully selected is apt to break and is too thick to use in the nose.

For convenience and safety in transportation "culture outfits" have been devised, which consist usually of a small wooden box containing a tube of blood serum, a tube holding a swab, and a record blank. These "culture outfits" may be carried or sent by messenger or express to any place desired.

*Directions for Inoculating Culture Tubes with the Exudate.*—The patient is placed in a good light, and, if a child, properly held. The swab is removed from its tube, and, while the tongue is depressed with a spoon, is passed into the pharynx (if possible, without touching the tongue or other parts of the mouth), and is rubbed gently but firmly against any visible membrane on the tonsils or in the pharynx, and then, without being laid down, the swab is immediately inserted in the blood-serum tube, and the portion which has previously been in contact with the exudate is rubbed a number of times back and forth over the whole surface of the serum. This should be done thoroughly, but it is to be gently done, so as not to break the surface of the serum. The swab should then be placed in its tube, and both tubes, thin cotton plugs having been inserted, are reserved for examination or sent to the laboratory or collecting station (as in New York City). If sent to the Health Department laboratories for examination the blank forms of report which usually accompany each "outfit" should be filled out and forwarded with the tubes.

Where there is no visible membrane (it may be present in the nose or larynx) one swab should be rubbed over the mucous membrane of the pharynx and tonsils, and another in the nasal cavities, and a culture made from these. In very young children it should be remembered that the throat often contains food or vomited matter. This should be cleared away before using the swab to make the bacteriological examination easier. Under no conditions should any attempt be made to collect the material shortly after the application of strong disinfectants (especially solutions of corrosive sublimate) to the throat. Cultures from the nostrils are often more successful if the nostrils are first cleansed with a spray of sterile normal salt solution.

*Examination of Cultures.*—The culture tubes which have been inoculated, as described above, are kept in an incubator at 37° C. for at least twelve hours, and are then ready for examination. When great haste is required, even five hours will often suffice for a sufficient growth of bacteria for a skilled examiner to decide as to the presence or absence of the bacilli. The absence of bacilli at this period cannot be relied upon. In primary cultures it is wise to reincubate tubes taken out under sixteen hours in which no bacilli were found. A small percentage of these will yield positive results after a few hours' further incubation. On inspection it will be seen that the surface of the blood serum is dotted with numerous colonies, which are just visible. No diagnosis can be made from simple inspection; if, however, the serum is found to be liquefied or

shows other evidences of contamination the examination will probably be unsatisfactory.

In order to make a microscopic preparation, a clean platinum needle is inserted into the tube and quite a large number of colonies are swept with it from the surface of the culture medium, a part being selected where the most suitable colonies are found. A sufficient amount of the bacteria adherent to the needle is washed off in a tiny droplet of water previously placed on the glass slide and smeared over its surface. The bacteria on the glass are then allowed to dry in the air. The glass slide is then passed quickly through the flame of a Bunsen burner or alcohol lamp, three times in the usual way, covered with a few drops of Löffler's solution of alkaline methylene blue, and left without heating for five to ten minutes. It is then rinsed off in clear water, dried, and mounted in balsam. When other methods of staining are desired they are carried out in the proper way (see Methods of Staining).

In the great majority of cases one of two pictures will be seen with the  $\frac{1}{2}$  oil-immersion lens—either an enormous number of characteristic Löffler bacilli, with a moderate number of cocci, or a pure culture of cocci, mostly in pairs or short chains. (See Streptococcus.) In a few cases there will be an approximately even mixture of Löffler bacilli and of cocci, and in others a great excess of cocci. Besides these there will be occasionally met preparations in which, with the cocci, there are mingled bacilli more or less resembling the Löffler bacilli. These bacilli, which are usually of the pseudodiphtheria type of bacilli (see Fig. 122), are especially frequent in cultures from the nose.

In not more than one case in twenty will there be any serious difficulty in making the diagnosis, if the serum in the tube is moist and has been properly inoculated. In the doubtful case another culture must be made or the bacilli plated out and tested in pure culture.

*Direct Microscopic Examination of the Exudate.*—An immediate diagnosis without the use of cultures is often possible from a microscopic examination of the exudate. This is made by smearing a slide or cover-glass with a little of the exudate from the swab, drying, heating, staining, and examining it microscopically. This examination, however, is much more difficult, and the results are more uncertain than when the slides are prepared from cultures. The bacilli from the membrane are usually less typical in appearance than those found in cultures, and they are mixed with fibrin, pus, and epithelial cells. They may also be very few in number in the parts reached by the swab, or bacilli may be found which closely resemble the Löffler bacilli in appearance, but which differ greatly in growth and in other characteristics, and have absolutely no connection with them. When in a smear containing mostly cocci a few of these doubtful bacilli are present, it is impossible either to exclude or to make the diagnosis of diphtheria with certainty. Although in some cases this immediate examination may be of the greatest value, it is not a method suitable for general use, and should always be controlled by cultures. When carried out in the best manner an experienced bacteriologist may obtain markedly accurate results. Higley,



in a series of consecutive throat cases, made the same diagnosis from the direct examination of smears as the Health Department laboratory made from the culture. To get the exudate he used a probe armed with a loop of heavy copper wire which had been so flattened as to act as a blunt curette. He then made thin smears from the exudate. After drying and fixing by heat the smears were stained for five seconds in a solution made by adding five drops of Kühne's carbolic methylene blue to 7 c.c. of tap-water. After washing and drying they were stained for one minute in a solution of 10 drops of carbol-fuchsin in 7 c.c. of water. The dilute solution should be freshly prepared. The diphtheria bacilli will appear as dark red or violet rods, and their contour, mode of division, and arrangement are manifest.

*Animal Inoculation as a Test of Toxicity.*—If the determination of the toxicity of the bacilli found is of importance, animal inoculations must be made. Experiments on animals form the only method of determining with certainty the toxicity of the diphtheria bacillus. For this purpose alkaline broth cultures of forty-eight hours' growth should be used for the subcutaneous inoculation of guinea-pigs. The amount injected should not be more than one-fifth per cent. of the body weight of the animal inoculated, unless controls with antitoxin are made. In the large majority of cases, when the bacilli are toxic, this amount causes death within seventy-two hours. If a good growth is not obtained in nutrient bouillon, ascitic broth should be used. At the autopsy the characteristic lesions already described are found. Bacilli which in cultures and in animal experiments have shown themselves to be characteristic may be regarded as true diphtheria bacilli, and as capable of producing diphtheria in man under favorable conditions.

For an absolute test of specific toxicity antitoxin must be used. A guinea-pig is injected with antitoxin, and then this and a control animal, with 2 c.c. of a broth culture of the bacilli to be tested; if the guinea-pig which received the antitoxin lives, while the control dies, it was surely a diphtheria bacillus which killed by means of diphtheria toxin—or, in other words, not simply a toxic bacillus, but a toxic diphtheria bacillus. Quite a number of bacilli have been found which kill 250-gram guinea-pigs in doses of 2 to 15 c.c., and yet are unaffected by antitoxin. These bacilli, though slightly virulent to guinea-pigs, produce no diphtheria toxin, and so cannot, to the best of our belief, produce diphtheria in man (see p. 301). The intradermal test may be used satisfactorily by the trained worker in testing toxin and antitoxin, resulting in the saving of the number of pigs used in the test. Rabbits can also be used in place of guinea-pigs.

**The Schick Reaction.**—Recently Schick published a method by which the presence of antitoxin in the blood and tissue can be determined very easily. A minute quantity of toxin is injected intracutaneously, and a local reaction follows if there is less than  $\frac{1}{30}$  of a unit of antitoxin per cubic centimeter of blood. This amount is considered sufficient to protect against diphtheria. The explanation of the test is that when no

antitoxin is present the toxin acts on the skin; when antitoxin is present it neutralizes the toxin so that no poisoning occurs. A negative reaction therefore indicates the presence of antitoxin.

A standard diphtheria toxin is diluted at first 1 to 10 in 0.5 per cent. phenol; this dilution will keep in the ice-box with little deterioration for at least two weeks. For use further dilutions are made in normal saline, of such strength that 0.1 c.c. contains  $\frac{1}{50}$  of the minimum lethal dose for the guinea-pig. This amount is injected intracutaneously on the flexor surface of the arm or forearm. If the injection has been made properly a definite wheal appears which lasts for several minutes. It is of the utmost importance that the injection be made in and not under the skin.

A positive reaction appears in twenty-four to thirty-six hours, and is characterized by a circumscribed area of redness and slight infiltration which measures from 1 to 2 cm. in diameter. It persists for seven to ten days, and on fading shows superficial scaling and a persistent brownish pigmentation. The test represents a true irritant action of non-neutralized toxin. Pseudoreactions are seen occasionally in young children and rather frequently in adults, who may have a large amount of antitoxin. These are local sensitization phenomena of a general protein character, and can be distinguished from the true reaction. They appear earlier, are more infiltrated, less sharply circumscribed and disappear in twenty-four to forty-eight hours. On fading they leave a faintly pigmented area which may show superficial scaling. They can also be obtained with the neutralized toxin and at times with dilutions of plain broth.

By the use of this test a large number of individuals have been shown to be naturally immune. Negative reactions were obtained in 93 per cent. of the newborn, in 57 per cent. during the first year of life, in 37 per cent. between two and five years, and 50 per cent. between five and fifteen years. In adults the negative reactions were as high as 90 per cent. By applying the test, therefore, passive immunization with antitoxin of a large number of individuals can be omitted and the disagreeable symptoms of sensitization avoided. Of 400 cases of scarlet fever, showing a negative Schick reaction and receiving no immunizing dose of antitoxin, not one developed clinical diphtheria. Of these cases 25 per cent. were carriers of virulent diphtheria bacilli.

An early series of cases in institutions gave the results shown in the table below.

SUMMARY OF SCHICK TESTS IN 2700 NORMAL CHILDREN.

	Total.	-Schick	+Schick.	Per cent. +Schick.
2 to 4 years . . . .	62	42	20	32.2
4 to 6 years . . . .	318	236	82	25.7
6 to 8 years . . . .	444	347	97	21.8
8 to 10 years . . . .	597	462	135	22.6
10 to 12 years . . . .	584	459	125	21.4
12 to 14 years . . . .	506	416	90	17.7
14 to 16 years . . . .	189	158	31	16.4
	<hr/> 2700	<hr/> 2120	<hr/> 580	<hr/> 21.4

From these figures, then, we may assume that the percentage of individuals susceptible to diphtheria is greatest between the ages of one to four years. It is less during the second six months of life and less in older children and in adults. Our results in scarlet fever cases closely approach those obtained by Schick, whose table shows positive reactions in 7 per cent. of the newborn, in 43 per cent. during the first year of life, in 63 per cent. between two and five years and 50 per cent. between five and fifteen years. In adults the positive reactions were not more than 10 per cent.

**Toxin-antitoxin Inoculations.**—Behring was the first who emphasized the practical application of toxin-antitoxin mixtures in which the toxin had been so neutralized as to be no longer poisonous, but still retained some toxin in loose combination. The use of this immunizing agent is the result of a long series of investigations as to the possibility of producing antitoxin through injection of these mixtures. In 1895 Babes carried out successful experiments in guinea-pigs. Since 1897 the horses in the Health Department of New York City, which are used to produce diphtheria antitoxin, have been immunized at first with these neutral mixtures. In 1903 one of us published the records of a number of horses showing that three injections might cause production of several hundred units of antitoxin in each cubic centimeter of serum. Theobald Smith later made a careful study of the subject in guinea-pigs and suggested the toxin-antitoxin injections in children for practical immunization but never tried it.

Behring deserves, therefore, the credit not of the discovery of the method but of actually applying the toxin-antitoxin mixtures for the immunizing of persons against diphtheria.

We have personally watched the result in a series of over 1000 cases, that had been actively immunized with diphtheria toxin-antitoxin. These susceptible individuals were selected by means of the Schick test out of a total of about 10,000 children and adults in 10 different institutions.

The mixtures of toxin-antitoxin that were used for immunization contained about 2L+ doses of toxin to each cubic centimeter, and were either neutral (66–70 per cent. L+ to each unit of antitoxin) or slightly toxic (80–90 per cent. L+ to each unit of antitoxin) to the guinea-pig. The dose was varied from 0.5 c.c. to 1.0 c.c., and the number of injections from one to three. The injections were made subcutaneously at intervals of seven days. The local reactions at the site of injection were generally mild; in the older children and adults, the redness and swelling were more marked. General symptoms, like malaise, and temperature of 100°–102° F., were noted in 10 to 20 per cent. of the cases; in a few the temperature reached 104° F. The symptoms lasted twenty-four to forty-eight hours, and then rapidly subsided. Both local and general symptoms were especially evident in those who showed a susceptibility to the protein by giving a combined pseudo and true Schick reaction. No harmful after-effects were noted in several thousand injections.

The retests with the Schick reaction showed that only 30 to 40

per cent. became immune three weeks after the first injection; about 50 per cent. at four weeks, 70 to 80 per cent. at six weeks, and 90 to 95 per cent. at eight to twelve weeks. The best results were obtained with the full immunization, consisting of three injections of 1 c.c. each, given at weekly intervals. The duration of the active immunity was studied in a group of children that was followed up for over one and one-half years; these cases showed that the active immunity persisted for at least that length of time. It is possible that the immunity induced by the injections of toxin-antitoxin starts a continued cellular production of antitoxin which otherwise would have appeared much later in life.

From their results Park and Zingher conclude that it is advisable to immunize children soon after the first year of life so as to afford them a protection against diphtheria at a time when the disease is most dangerous. In addition such young children, by not having any hypersensitiveness to the bacillus protein, show very mild local and constitutional symptoms after the injections. An immune child population could thus be developed with the result that fresh clinical cases would be prevented and the bacillus-carrier menace would probably soon disappear as a hygienic factor in our communities.

Interesting and parallel results were noted in guinea-pigs and horses. *Guinea-pigs* are fairly resistant to active immunization with diphtheria toxin-antitoxin, and in that respect they show an almost complete parallelism to the positive Schick cases among human beings. After injections of toxin-antitoxin, an antitoxic immunity develops slowly from the sixth to eighth week. *Horses*, on the other hand, as a rule, correspond to those human beings who are naturally immune in their behavior toward small doses of toxin-antitoxin. They both give a ready response, even after a single injection of toxin-antitoxin, and show a distinct increase in the antitoxin content toward the end of the first week. Occasionally a horse is found that has no antitoxin in the control bleeding; such animals respond slowly to small doses of toxin-antitoxin. It is probable that the tissue cells of the naturally immune human beings and the majority of horses have acquired the property of giving a quick and easy response to the stimulation of diphtheria toxin.

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## CHAPTER XXI.

### INTESTINAL BACTERIA.

#### SOUR MILKS.

**Conditions Influencing Bacterial Development.**—The alimentary tract both as regards foodstuff, varying degrees of oxygen tension and reaction gives so many different conditions for growth that many varieties of bacteria find an optimum environment at some level. Thus, the mouth offers, in general, an aërobic area, although with its various crevices between the teeth, in the folds of the mucous membranes and the crypts of the tonsils, anaërobic types may flourish with aërobic types, which absorb the oxygen. The stomach when its acidity is normal, destroys bacteria unless protected by food particles. As soon as the duodenum is reached the oxygen tension becomes low and from there on an anaërobic condition exists. More aërobic conditions are reached in the lower colon and rectum. It is evident that the degree of digestion of the food and the proportion of unabsorbed carbohydrate and protein at different levels will also influence the flora, likewise the products of bacterial growth at one level will influence the growth of other types at this and at lower levels. As the intestinal contents pass downward, they carry with them the bacteria from a higher level, but if the conditions are not favorable for their growth they are quickly overshadowed by the more adapted types. In the lower part of the large intestine and in the rectum due to the gradual loss of water, there is marked tendency of the bacteria in fecal mass to die or to become so attenuated as to be incapable of further growth.

**Development of the Intestinal Flora.**—At birth the meconium is sterile unless fetal infection has taken place due to general infection in the mother. Shortly after birth bacteria and yeasts are found entering either through the rectum or by way of the mouth from swallowing saliva or food. Very soon, through further ingestion of bacteria and by multiplication of different types at their optimum levels, a more or less distinctive bacterial flora is established, distinctive in that the breast-fed infant, the artificially fed infant and the adolescent or adult each has, considering the dominant types, a somewhat characteristic flora. This flora is susceptible, however, to variation through the administration of cathartics, by changes in diet, by implantation through feeding of cultures or during alimentary infections.

**Importance of the Intestinal Flora.**—Whether the development of such an intestinal flora is of physiological advantage to the host is open to question. Successful experiments in raising animals with a sterile intestinal tract show that such a flora is not a physiological

necessity. Experiments in which animals so raised did not do well, cannot be considered evidence against this view, as the conditions of the experiment may have deleteriously influenced development. Undoubtedly the character and balance of the flora does act as a protection under some circumstances, being antagonistic to the implantation of an exogenous pathogenic type. Abnormal variations, however, in balance between the dominant types may be the basis of intestinal symptoms (see below). In one sense part of the intestinal flora is a potential menace to the host. The intestinal mucosa is an obstacle to the passage of the bacteria into the tissues, lymph, and blood. A few, however, evidently escape as the fairly frequent localization in the gall-bladder, kidney and elsewhere show. Probably this occurs constantly in small numbers, but because of the slight virulence of the majority of such types they are promptly disposed of.

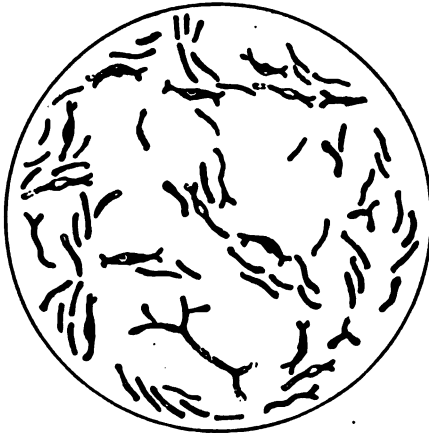


FIG. 124.—*B. bifidus*, representing the various forms described; the irregularly stained or vesicular forms being from old cultures.  $\times$  about 1800 diameters.

**Dominant Intestinal Bacterial Types.**—*B. bifidus*.—A strict anaërobe isolated by Tissier from the stools of breast-fed infants and from the superficial ducts of the mammary glands of mothers. It is Gram-positive or contains a Gram-positive granule, the remainder of the bacillus being Gram-negative. In stools it is a slender bacillus with one end tapering, the other club-shaped. In cultures it has the property of developing bifid ends. It produces acid freely but no gas from lactose or other sugars.

**B. Acidophilus.**—A type of bacterium characterized by its acid tolerance. *B. bulgaricus* (see below) is related to this type, both being included in the aciduric group. It is a Gram-positive, non-spore-bearing pleomorphic bacillus, frequently forming chains in cultures and producing acids freely from carbohydrates. A similar gas-producing type has been described.

**Enterococcus or Micrococcus Ovalis.**—A Gram-positive, oval coccus, usually in pairs or chains. Capsules may be present. Due to delayed

cleavage pseudobacillary types may develop on certain media. It is aërobic, facultative anaërobic, non-liquefying, producing acid from glucose and lactose, and coagulating milk.

**B. Putrificus.**—An anaërobic slender, Gram-positive, motile putrefactive bacillus resembling *B. tetani* in its morphology.

**B. Mesentericus.**—Like *B. subtilis*, it is essentially a Gram-positive, spore-bearing, aërobic, proteolytic bacillus, that is, forming products of decomposition in protein media when glucose is absent.

**B. (aërogenes) capsulatus.**—See p. 332.

**B. Coli and Allied Types.**—See pp. 323–334.

**Intestinal Flora of Breast-fed Infants.**—The *Micrococcus ovalis* types preponderate in the duodenum and are most numerous when digestion is in progress. The dominant type in the remainder of the small intestine is *B. aërogenes*, *B. coli* being most numerous about the cecal level. *B. bifidus* finds its optimum in the large intestine. Putrefactive bacteria are uncommon in normal breast-fed infants. Fecal smears show a preponderance of Gram-positive types.

**Intestinal Flora of Artificially Fed Infants.**—The Gram-negative *B. coli* and *B. aërogenes* as well as the *M. ovalis* are relatively increased as compared with the breast-fed infant, while *B. bifidus* types are diminished, being replaced by colon types and *B. acidophilus*. Proteolytic action is evidenced in the alkaline reaction of the feces. In general the distribution of the types follows that in breast-fed infants. The character of the flora is susceptible to variation according to the balance of carbohydrate to protein and by changes in the carbohydrate employed.

**Intestinal Flora of Adults.**—The duodenum except during digestion has a low bacterial content. Cocci predominate in the upper small intestine. *B. coli* types become numerous in the lower small intestine, and together with *B. mesentericus* types predominate about the cecal level. These and, to a limited extent, the proteolytic anaërobic constitute the flora of the large intestine. The flora as compared with that of infants is characterized by the absence of essential carbohydrate-fermenting types. The carbohydrate of adult life is mostly starch and the products of its cleavage are probably quickly absorbed, leaving the more slowly cleaved and absorbed protein as available foodstuff for the bacteria. The colon types and the aërobic and anaërobic proteolytic types which can utilize this material therefore dominate the flora.

**Variations in the Bacterial Flora and Their Significance.**—The flora as given for the three types is basically dependent on the diet. Thus, in the nursing infant, the carbohydrate is sufficient in amount to supply the conditions for growth of the essentially fermentative types. These because of their acid production limit to a marked degree the multiplication of other types, especially the proteolytic types. Although in the case of the artificially fed infant the available carbohydrate is less in amount, it is still sufficient to lead to relatively the same result. If, however, the protein proportion of the food be increased, a relative or complete suppression of acid-producing types with an increase of the proteolytic types occurs, approximating the flora of the adult. The

activity of *B. coli* as regards end-products depends also on the availability of carbohydrates. If present in sufficient amounts, lactic acid and other end-products of carbohydrate fermentation are produced. When absent, the end-products of protein cleavage,  $H_2S$ ,  $NH_3$ , and indol, are produced. It is easy to see how carbohydrate fermentation acts as a check to excessive putrefactive activity, which condition is deleterious to the host. The bacterial flora, as a rule, is little influenced by the injection of bacteria in the foods.

When infection occurs, such as with dysentery or cholera, these organisms and others similar to them may dominate the intestinal flora and a specific disease develops. Apart from these well-known types of disease there are other morbid conditions which, although of unknown etiology, are intimately associated with changes of the intestinal flora, even if these changes are not of etiological importance. These can be subdivided as to whether (a) the products of bacterial proteolysis, (b) the products of carbohydrate fermentation or (c) both are at fault. The last is the least understood.

In the first case a condition of auto-intoxication develops with the presence of ethereal sulphates in the urine. In the second the production of abnormal or the excessive production of the products of fermentation as simple hyperacidity or less-known irritative products due to the overactivity of *B. aërogenes* or *B. capsulatus* are the probable cause of the symptoms. Diarrheal conditions due to the latter, even though chronic, do not show evidences of toxemia.

On this basis attempts have been made to influence these conditions. Cathartics, intestinal antiseptic and starvation have no appreciable beneficial value. Changes in diet so adapted as to limit or supply the optimum foodstuff for certain types or the addition to the diet of exogenous lactic acid-producing bacilli and the products of their growth have been more successful. The latter method first recognized by Herter has been popularized by Metchnikoff's propaganda of sour-milk therapy.

**Types of Sour Milk (Lactic Acid Milks).**—Milk will sour naturally due to lactic acid bacteria which are contaminations of the milk from its surroundings. Buttermilk is a naturally soured product. Metchnikoff advised the use of *B. bulgaricus* (see below) found in the sour milk of Eastern Europe and in Asia. Many lactic acid-milk preparations are available. To control the appearance of the end-product and to have a uniformly palatable preparation, it is necessary to heat the milk and add a starter. Thus "koumyss" is fermented by lactic acid bacteria and yeasts. "maadsoun" and "zoolak" by *B. bulgaricus* and lactic acid producing streptococci and diplococci. Many milk dealers now offer lactic acid milks under various trade names, the starters used being those mentioned in various combinations. *B. bulgaricus* alone gives too sour a milk.

**B. Bulgaricus.**—The types included under this name belong to the aciduric group of bacilli. They are non-motile, Gram-positive (except involution forms which are negative) aërobic, facultative anaërobic,



non-liquefying bacilli occurring singly or in chains. They require carbohydrates for their growth, growing best on or in milk or whey media, producing large amounts of acids, which, however, do not readily inhibit their growth. On whey agar, they produce characteristic colonies (see Fig. 125). Milk is quickly coagulated. White and Avery have differentiated two groups depending upon the amount of lactic acid produced.

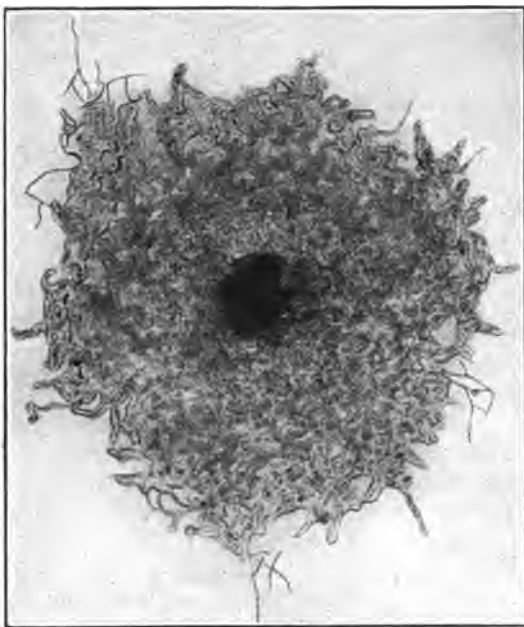


FIG. 125.—*B. bulgaricus*; seventh day (44°) colony. Whey agar plate.  $\times 50$  diameters. (White and Avery.)

**Other *B. Bulgaricus* Preparations.**—Other methods of using *B. bulgaricus* in the form of liquid cultures, dried tablets or oily suspensions are of less value than milk preparations. Their use is based on the assumption that the bacilli will be active in the intestinal tract, which is an assumption of doubtful validity. Furthermore, they do not supply the preformed acid, at least to any extent, as contained in milk preparations. Bendick has found that many of the tablets and probably the oily suspensions as well, contain very few viable organisms; and the bacilli in liquid preparations tend to die out rapidly. At best, this preparation does not compare with sour milk in its bacillary content.

In conditions of toxemia the limitation of proteins with liberal drinking of sour-milk preparations, flooding the intestinal tract with preformed acids is beneficial, but the implantation of *B. bulgaricus* as an intestinal type probably does not occur. For the latter purpose it would seem more logical to feed *B. acidophilus* which is naturally an intestinal

parasite. In the special fermentative types of diarrhea due apparently to an overgrowth by *B. capsulatus* the liberal use of acid buttermilk



FIG. 126.—*B. bulgaricus*.  $\times 1000$  diameters. (Piffard.)



FIG. 127.—“Lactic acid” milk containing *B. bulgaricus* and a lactose-fermenting streptococcus.

and a carbohydrate-free diet will lead to improvement. The same treatment should be applied if *B. aërogenes* is the apparent causative agent.

**The Influence of Available Foodstuffs in Specific Intestinal Infections.**—In infections due to members of the typhoid-dysentery group or to the cholera vibrio the presence or absence of available carbohydrates may have an important bearing on the products developed by these bacteria and thus on the disease. Each will probably utilize carbohydrate if available, in preference to proteins (protein sparing). There is no evidence that the acids produced from carbohydrate cleavage are any more harmful than those produced by any non-pathogenic fermentative organism. Furthermore, the acid produced would limit their further growth. Not only may the products of protein cleavage be harmful in themselves, but the toxicity of the products of growth, whether a free toxin is produced or not, is enhanced in amount when growth is due to the utilization of protein material.

A free carbohydrate diet, therefore, should be valuable in these diseases not only to spare protein but as an incentive to increased activity of the aciduric types which would aid by limiting multiplication. Although difficulties due to inflammatory conditions of the intestines and other causes may interfere with the efficiency of this mode of treatment, its results in general have been good. Coleman and Schaffer, with the high calory diet in typhoid fever, have not only demonstrated that the nitrogen and weight loss is prevented to a large extent, but that the toxemia is reduced. Liberal feeding of lactose in dysentery has yielded similar results.<sup>1</sup>

<sup>1</sup> For fuller discussion and bibliography see:

HERTER: *Bacterial Infections of the Intestinal Tract*, New York, 1907.

KENDALL: *Bacteriology—General, Pathological and Intestinal*, Philadelphia, 1916.

## CHAPTER XXII.

## THE COLON-TYPHOID GROUP OF BACILLI.

THERE are a number of varieties of bacilli normally occupying the intestines of man and animals which, because they have similar characteristics and live in the colon, are generally grouped together as colon bacilli. Many of the varieties occurring in animals are culturally like those found in man. These bacilli are only pathogenic under unusual conditions. The specific pathogens, typhoid, paratyphoid, including the types responsible for meat poisoning, dysentery, and paradysentery bacilli, also have among themselves and between them and the colon bacilli resemblances and are often classed together in the group of the colon-typhoid bacilli.

The chief characteristics common to this whole group are: (1) a similar morphology, *i. e.*, short, rather plump non-spore-bearing rods with a tendency to thread formation; (2) a Gram-negative staining reaction; (3) similar growths on agar and gelatin; (4) non-liquefaction of gelatin (a few closely related organisms, such as *B. cloacæ*, liquefy gelatin very slowly).

In order to see more clearly the main points of difference among the subdivisions of this great group the following tabulations may be studied.

### GROUP OF COLON-TYPHOID BACILLI.

Colon group . . . . .	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;"> B. (coli) communis  B. (coli) communior  B. (acidi) lactici  B. aërogenes types  B. paratyphosus, A  B. paratyphosus, B  B. paratyphosus, C  B. enteritidis </div> <div style="display: inline-block; vertical-align: middle; font-size: 3em; line-height: 1;">}</div> </div>	Normal inhabitants of the intestines, under certain conditions become pathogenic.
Paratyphoid group (intermediate group)	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;"> B. enteritidis </div> <div style="display: inline-block; vertical-align: middle; font-size: 3em; line-height: 1;">}</div> </div> <p>(Similar types, intermediates between colon and typhoid have been isolated from water and other sources, probably non-pathogenic.)</p>	Usually pathogenic for man or animals in varying degree.
B. typhosus . . . . .		Pathogenic for man.
Dysentery group . . . . .	<div style="display: inline-block; vertical-align: middle;"> <div style="display: inline-block; vertical-align: middle;"> B. dysenteriae (mannite not fermented).  B. paradysenteriae (mannite fermented).  Three varieties. </div> <div style="display: inline-block; vertical-align: middle; font-size: 3em; line-height: 1;">{</div> </div>	Pathogenic for man, paradysenteriae usually less so than dysentery type.
Alkaligenes group . . . . .	B. alkaligenes.	Occasionally pathogenic.

## ESSENTIAL DIFFERENTIAL REACTIONS—COLON-TYPHOID GROUP.

Lactose	{	Acid and gas (colonies, red on Endo and Conradi)	Glucose—acid and gas—Colon group. (Russell medium—slant, acid butt, acid and gas.)
	{	not fermented, (colonies, color- less on Endo, blue on Conradi)	Glucose—acid and gas—Paratyphoid-enteritidis group. (Russell medium—slant, unchanged butt, acid and gas.)
			Glucose—acid only— <i>B. typhosus</i> —dysentery group. (Russell medium—slant, unchanged butt, acid only.)
			Glucose—not fermented— <i>B. alkaligenes</i> . (Russell medium—slant, unchanged butt, unchanged.)

Among the non-lactose fermenters motility or its absence is of differential value. The fermentative characteristics of value in differentiating members of the subgroups are given with each subgroup.

**Russell's Medium.**—The reactions as given are based on the fact that the bacilli growing aërobically, that is, on the slant, only utilize the carbohydrate when present in amounts over 0.1 per cent., whereas growing anaërobically stabbed in the butt, they must utilize the carbohydrates for their oxygen supply and therefore ferment the trace of glucose present. By adding 1 per cent. saccharose intermediate types are differentiated.

## THE COLON GROUP.

The first description of an organism of the colon type was by Emmerich (1885), who obtained it from the intestinal discharges of cholera patients. A similar organism was found by Escherich (1886) in the feces of healthy infants. He gave it the name of *Bacterium coli commune*. It has since been demonstrated that closely allied types of bacilli are normal inhabitants of the intestines of most of the lower animals. They are transferred through the feces as manure and sewage to cultivated land, surface waters, etc. During warm weather they may multiply outside of the animal body. Those strains having the chief cultural characteristics of the original strains are classed as colon bacilli, while those differing considerably from it are, while considered in the general group, given different names, such as paracolon, "atypical coli," etc. These types are especially common in water and their atypical reactions are often due to the suppression of certain characteristics due to unfavorable environment.

The colon group is divided into four main subgroups, *B. communis*, *B. communior*, *B. aërogenes*, and *B. (acidi) lactici*. These subgroups have numerous varieties.

The colon group has interest not only because it excites disease at times in man and animals, but also because it is an index of fecal pollution from man or animals. If from man it indicates the possibility of

infection with the typhoid or dysentery bacilli. For the significance of the colon group in water see Bacteriology of Water.

**B. (Coli) Communis.**—This organism is taken as the *type* of the group in the following description. Only differentiating points are noted in the other varieties.

**Morphology.**—*B. communis* varies in morphology. The typical form (Fig. 128) is that of short rods with rounded ends, from  $0.4\mu$  to  $0.7\mu$  in diameter by  $1\mu$  to  $3\mu$  in length; sometimes, especially when the culture media are not suitable for their growth and in tissues, the rods are so short as to be almost spherical, resembling micrococci in appearance, and, again, they are somewhat oval in form or are seen as threads of  $6\mu$  or more in length. The various forms may often be associated in the same culture. The bacilli occur as single cells or as pairs joined end-to-end, rarely as short chains. There is nothing in the morphology of this bacillus sufficiently characteristic for its identification.



FIG. 128.—Colon bacilli. Twenty-four-hour agar culture.  $\times 1100$  diameters.

**Flagella.**—Upon some varieties seven or eight peritrichic flagella have been demonstrated, the non-motile types show none. The flagella are shorter and more delicate than those characteristic of the typhoid bacilli.

**Staining.**—The *B. communis* stains readily with the ordinary aniline colors; it is always decolorized by Gram's method. Under certain conditions the stained bacilli exhibit bipolar granules.

**Biology.**—It is an *aërobic, facultative anaërobic, non-liquefying* non-spore-bearing bacillus. It develops best at  $37^{\circ}\text{C}.$ , but grows well at  $20^{\circ}\text{C}.$ , and slowly at  $10^{\circ}\text{C}.$  It is usually motile, but the movements in some of the cultures are so sluggish that a positive opinion is often difficult. In fresh cultures, frequently only one or two individuals show motility.

**Cultivation.**—The *B. communis* develops well on all the usual culture media. Its growth on them is usually more abundant than that of the

typhoid bacillus or the dysentery bacillus, but the difference is not sufficient for a differential diagnosis.

**Gelatin.**—In gelatin plates, colonies are developed in eighteen to thirty-six hours. They resemble greatly the colonies of the typhoid bacillus, except that many of them are somewhat larger and more opaque. (See Figs. 43 to 45, page 115.) When located in the depths of the gelatin and examined by a low-power lens they are at first seen to be finely granular, almost homogeneous, and of a pale yellowish to brownish color; later they become larger, denser, darker, and more coarsely granular. In shape they may be round, oval, or whetstone-like. When the gelatin is not firm the margins of many colonies are broken by outgrowths, which are rather characteristic of colon bacilli.

In stab cultures in gelatin the growth usually takes the form of a nail with a flattened head, the surface extension generally reaching out rapidly to the sides of the tube.

**Nutrient Agar.**—In plate cultures: Surface colonies mostly circular, finely granular, and rather opaque. The deep colonies are likely to have protuberances. In streak cultures an abundant soft, white layer is quickly developed, but the growth is not characteristic.

**Bouillon.**—In bouillon the *B. communis* produces diffuse clouding with sedimentation; in some cultures a tendency to pellicle formation on the surface is occasionally seen.

**Potato.**—On potato the growth is rapid and abundant, appearing after twenty-four to thirty-six hours in the incubator as a yellowish brown to dark cream-colored deposit covering the greater part of the surface. But there are considerable variations from the typical growth; there may be no visible growth at all, or it may be scanty and of a white color. These variations are often due to variations in the potato.

**Milk.**—Milk is usually coagulated in from one to four days at 37° C. Coagulation is due principally to the production of lactic and acetic acid. Some strains produce acid but coagulation does not occur. It is possible that a lab ferment is partly responsible for the coagulation.

**Chemical Activities.—Behavior Toward Carbohydrates.**—In cultures of *B. communis* many carbohydrates, especially sugars, become fermented with production of acid and gas.

The important fermentation products, both qualitatively and quantitatively, are produced from grape-sugar, probably according to the following reaction:



There are reasons to think that lactic acid is first produced and that from this other acids and products develop. Under aerobic conditions lactic acid is produced in excess of acetic, while in the absence of oxygen the reverse is likely to be true.

**Gas Production.**—When *B. communis* is grown in a solution of glucose (dextrose),  $\text{CO}_2$  and  $\text{H}_2$  are produced, in the proportion of  $1\text{CO}_2$  to  $1\text{H}_2$

up to  $1\text{CO}_2$  to  $3\text{H}_2$ . Anaërobic conditions aid gas formation. Very slight traces of gases other than  $\text{H}_2$  and  $\text{CO}_2$  are produced. The amount of gas varies in different varieties; the closed arm of the tube half-filled, and the  $\text{H}_2$  and  $\text{CO}_2$  in the proportion 2 to 1, is the characteristic type.

The fermentation is not a simple hydrolytic action, but one in which combinations between the C and O atoms are sundered and formed. This is not an oxidation process, but a change through breaking down—that is, a true decomposition. What oxidation takes place is chiefly due to the oxygen liberated from splitting the sugar molecules.

The fermentative reactions of the main subgroups based on the work of Smith and of MacConkey are as follows:

- B. (coli) communis—saccharose, negative—dulcit, positive.
- B. (coli) communior—saccharose, positive—dulcit, positive.
- B. (acidi) lactici—saccharose, negative—dulcit, negative.
- B. aërogenes—saccharose, positive—dulcit, negative.

Kligler found that a subdivision according to the fermentative reactions in salicin correlated more fully with other characteristics and suggests the following classification:

- B. communis—saccharose, negative—salicin, positive  
(dulcit usually positive).
- B. communior—saccharose, positive—salicin, negative  
(dulcit usually positive).
- B. lactici—saccharose, negative—salicin, negative  
(dulcit usually negative).
- B. aërogenes—saccharose, positive—salicin, positive  
(dulcit usually negative).

The last group usually gives a positive Voges-Proskauer reaction (see Bacteriology of Water).

**Effect of B. Communis in Nitrogenous Compounds.—Indol Formation.**—*B. communis* does not liquefy gelatin nor peptonize any albumins. They do, however, break down some of the higher nitrogenous compounds into smaller atom groups. The first noted of these compounds was indol,  $\text{C}_6\text{H}_5\text{NHCH}$ . This is one of the most important products of

colon activity, although some varieties lack the ability to produce it. The maximum amount of indol is present about the tenth day. In the intestinal canal in health very little indol appears to be produced by the bacilli. Sulphurated hydrogen is liberated from sugar-free protein substances.

**Reduction Processes.**—Nitrates are reduced to nitrites and from them ammonia and free nitrogen. Litmus and other dyes are also reduced.

**Toxins.**—The bodies of dead bacilli contain pyogenic and other substances, which, injected into the circulation, produce paralysis of the striped muscle fibers, convulsions, coma, and death. Extracts from some cultures produce irritation of the mucous membranes of the large intestines with dysenteric symptoms.



**Growth with Other Bacteria.**—The *B. communis* as well as other members of the colon group act antagonistically to many of the proteolytic bacteria in the intestinal tract, and so inhibit alkaline putrefaction otherwise caused by the latter. In milk the same antagonism exists, probably because of the acidity caused by the colon growth.

**Reaction to High and Low Temperatures.**—*B. communis* is killed at 60° C. in from five to fifteen minutes. Frozen in ice a large proportion die, but some resist for six months. Frozen in liquid air 95 per cent. are killed in two hours.

**Resistance to Drying and Antiseptics.**—Simply drying destroys the majority of organisms dried at any one time, but some bacilli of the number dried may remain alive, especially when held in the texture of threads, for five or six months, or all may die in forty-eight hours.

To most antiseptics they are moderately resistant. They are killed in five to fifteen minutes in a 1 per cent. solution of carbolic acid.

**Effect of Acids.**—*B. communis* grows in a wider range of acids and alkalis than most other bacteria. It develops in from 0.2 to 0.4 per cent. of mineral acids, in from 0.3 to 0.45 per cent. of vegetable acids, and in from 0.1 to 0.2 per cent. of alkalis.

**Effect of Intestinal Juices.**—Gastric juice kills unprotected *B. communis* unless it is too greatly diluted by food. All the members of the typhoid-colon group are more resistant to the gastric juices than most non-spore-bearing bacteria. With the food they readily pass from the stomach into the intestines. They grow in bile and in the intestinal juices.

**Pathogenesis.**—**In Lower Animals.**—Intraperitoneal and intravenous inoculation of guinea-pigs and rabbits may produce death, which, when it follows, usually takes place within the first forty-eight hours, accompanied by a decided fall of temperature, the symptoms of enteritis, diarrhea, etc., and finally fibrinopurulent peritonitis.

Subcutaneous inoculation into rabbits is followed usually by abscess formation at the point of inoculation. Dogs and cats are similarly affected.

Cystitis and pyelonephritis may be produced by direct injections into the bladder and ureters, if the urine is artificially suppressed. Angiocholitis and abscess are produced by direct injections into the liver. Osteomyelitis may follow the intravenous injections of cultures in young rabbits.

From experiments on animals it would appear that the explanation of the pathogenesis of the colon bacillus is undoubtedly to be found in the toxic effects of the chemical substance and products of the cells.

**In Man.**—In normal intestines with intact mucous membranes the toxic products formed by *B. coli* are absorbed but little or not at all, and the bacilli themselves are prevented from invading the tissues by the epithelial layer and the bactericidal properties of the body fluids. Possibly there is an acquired immunity to the colon varieties which have long inhabited the intestines.

*B. coli* was at first regarded purely as a saprophyte. Later, because of the postmortem invasion and the great ease of growth of the colon bacillus on ordinary media, the other extreme of attributing too much to it was taken.

The bacilli present in the intestines may, either by an increase in virulence or by a lowered resistance in the person, cause intestinal inflammation or more distant infections. Thus in the case of ulceration in typhoid fever *B. coli* may produce peritonitis. In conditions approaching death they at times pass through the intact mucous lining. The spread of bacilli from the intestines may also cause disease in the gall-bladder or urinary tract. The specific serum reaction in the body is a sign of infection, but great care has to be observed in deciding that it is present, as group agglutinins also occur. Up to the present time it is very difficult to state in any colon infection whether the bacilli were previously present in the intestines or were derived from outside sources through water, food, or direct contact with other cases.

**Intestinal Lesions.**—The lesions present in intestinal inflammation attributed to *B. coli* are those of enteritis; the duodenum and jejunum are found to contain fluid, the spleen is somewhat enlarged, and there are marked hyperemia and ecchymosis of the small intestines, together with swelling of Peyer's patches.

**Virulence of *B. Coli* from Normal and Diseased Intestines.**—The virulence varies with the culture and the time since its recovery from the intestines. Other things being equal, it is usually more virulent from an intestinal inflammation. From severe diarrhea the colon bacilli in 0.25 c.c. bouillon culture may kill guinea-pigs if given intraperitoneally, while from the healthy bowel 2.5 c.c. are usually required.

***B. Coli* in Sepsis.**—When lesions of the intestinal mucous membranes exist, or in colon cystitis, pyelitis, or cholecystitis, there is frequently just before death a terminal dissemination of the bacilli and consequent septicemia. The colon septicemia is detected by blood cultures. At times very few bacilli are found, and then the blood infection may be less important than the local one. Cases occurring in typhoid and cholera have been observed, especially in relapses in typhoid. In very young infants a malignant septicemia with tendency to hemorrhages may be due to *B. coli*. In a few cases in which *B. coli* but no typhoid bacilli were present the course of the disease has been similar to typhoid fever. An epidemic probably due to colon infection of water has been noted. Infections through food and water are usually caused by other closely allied bacilli not belonging to the colon group.

***B. Coli* in Diarrhea.**—In diarrhea we find increased peristalsis, less absorption of foodstuff, increased and changed intestinal secretions. Tissier observed that under treatment with cathartics the colon varieties increased, while the anaërobic forms are inhibited. In diarrhea, therefore, we should expect favorable conditions for multiplication with the inhibiting causes lessened. This makes one question the importance of the significance of numerous epidemics which have been reported of acute diarrhea in children from one to five years of age in which almost

pure cultures of colon bacilli have been found. The symptoms in such epidemics begin with high fever which often rapidly falls, and frequent stools, only watery or containing mucus and streaks of blood. These symptoms may quickly abate or go on to a toxic state characterized by heart weakness and drowsiness. This may lead to lung complications or death.

**B. Coli in Peritonitis.**—Here the lesions must be considered as being due to mixed infection. Not only perforation of the intestines in man, but injury to the intestinal walls, allows colon infection of the peritoneum to take place. At first most of these cases were believed to be a pure colon infection, but now it is known that this idea came largely from the overgrowth of colon bacilli in the cultures. More careful investigations, through cultures and smears, have demonstrated the fact that streptococci, and less frequently staphylococci and pneumococci, are also usually present in peritonitis arising from intestinal sources. The colon bacilli found even in the same case commonly comprise many varieties.

**B. Coli in Inflammation of the Bile Tract.**—The normal healthy gall-bladder is usually sterile. This is true in spite of the fact that bile is apparently a good culture medium for the colon group. Ligation of the neck of the gall-bladder usually causes a colon infection to take place within twenty-four hours. Obstruction of the bile-duct through various causes is fairly common in man. The gall-bladder then becomes infected, and following the inflammation of the mucous membranes there is often the formation of gall-stones. Some cases of jaundice are believed to be due to colon inflammation of the gall-ducts. Atypical varieties of *B. coli* are frequently isolated from gall-bladder infections.

**Inflammation of the Pancreas.**—Welch was the first to record a case of pancreatitis with multiple fat necroses due to *B. coli* infection. A few more cases have since been reported due to members of the colon group, either alone or in conjunction with the pyogenic cocci.

**Inflammation of the Urinary Tract.**—As far back as 1879 Bouchard noted cystitis due to bacilli of the colon group. When cystitis is established the bacterial infection frequently spreads to the pelvis of the kidneys, causing a pyelitis or suppurative nephritis. In most cases of chronic cystitis the ureters and pelves of the kidneys become involved; any malformation of the ureters aids the process. From the pelvis the bacteria push up into the urinary tubules and excite inflammation and multiple abscesses. Colon infection of the different parts of the urinary tract may occur at any age, from infancy upward. Instead of starting in the bladder it may begin in the kidney itself, the colon bacilli coming from the blood or peritoneum. In many of these cases the bacilli isolated from the urine are agglutinated in high dilutions of the blood from the patient.

Although other bacteria—the pyogenic cocci, the proteus, the typhoid bacillus, etc.—may excite cystitis, still in 90 per cent. of all cases some of the colon group are found, and this percentage is even higher in young children. The clinical picture of colon infection is very variable. The

lightest cases progress under the guise of a bacteriuria. The urine is passed a little more frequently and shows a fine granular cloudiness. The reaction is acid. The cell elements are but little increased. There is an excess of mucus. Albumin is absent or present in only a trace. The condition may last for weeks or months and then spontaneously disappear or grow worse. With a somewhat more severe infection there is painful urination, perhaps tenesmus, increase of pus cells and slight fever. In a conical glass a sediment of pus cells forms at the bottom, and clear urine remains above. If the infection passes to the kidney colicky pain and tenderness over the region of the kidneys is usually present. The most important symptom of pyelitis is an irregular intermittent fever resembling malaria. The albumin is increased in the urine and red blood cells may be seen. If a general nephritis arises the symptoms are all intensified and an anemic condition may develop. Septicemia may finally result.

In most of these cases the microscopic examination is sufficient to make a probable diagnosis, since the bacteria are so abundant. The variety of colon bacillus present can, of course, only be told by cultures and other means. In the urine they appear as diplobacilli, or partly in short, almost coccus, forms, partly in long threads. As a rule motility is absent. Not infrequently the cultures appear to be identical with those of the *Bacillus aërogenes*.

The characteristics of the urine itself have much to do with the probability of infection; the more acid urines being less likely to afford a proper soil for growth. Some urines are bactericidal even when they are neutral. The substances producing this condition are not known. The colon bacilli in the urine produce no appreciable effect on the reaction, but give up some of their toxins, which upon absorption cause the deleterious local and general effects. The serum of the patient usually agglutinates the cultures from the urine in 1 to 20 or 1 to 50 dilutions, but this property is sometimes absent, especially in light cases.

In all cases in addition to the introduction of the colon bacillus a predisposing condition must be present, such as more or less marked retention of urine by an enlarged prostate or stricture, any unhealthy state of the mucous membrane or general depression of vitality.

**B. Coli as Pus Former.**—Members of this group are frequently the cause of abscesses in the region of the rectum, urethra, and kidney. They rarely produce pus in other locations.

**B. Coli in Inflammation Not Previously Mentioned.**—Broncho-pneumonia, lobar pneumonia, and pleurisy have occasionally been caused by colon bacilli, probably from blood sources. Not a few cases of meningitis and spinal meningitis in infants, follow localized *B. coli* infections. The symptoms are not well developed as a rule. Some cases of endocarditis and of conjunctivitis have also been noted.

**Immunity.**—Natural infection in man, or inoculation into animals, is followed by the production of antibodies; agglutinins, precipitins, bactericidal substances, and opsonins being produced. The attempts to separate the members of this group by agglutination have shown the

great dissimilarity of the different members in their immune reactions, although group reactions are marked. Natural agglutinins for this group are commonly present in the serum of man and animals. (See Agglutination.)

**Vaccine Therapy.**—See Part III.

**Methods of Isolation.**—They may be isolated from lesions on ordinary media, in bile media or on Endo or Conradi plates. The latter are used in isolation from feces or other mixed material. Blood cultures may be made in bile or broth. For the examination of water for members of the colon group, see Bacteriology of Water.

**Bacillus Aërogenes and Allied Encapsulated Bacilli.**—The members of this group differ from the members of the colon group, already described, in producing a viscid or mucoid growth, and in smears a capsule is commonly demonstrable. For this reason the group is spoken of by some as the capsulatus group, and the aërogenes types described as allied varieties. There are wide variations in the group, not only in the degree of capsule production but in the ability to ferment different carbohydrates. In the absence of an accepted biological classification of types, the varieties are best considered according to their source. For this reason the term *B. aërogenes* is collectively used for the types normally found in feces, milk, etc.

**B. Aërogenes.**—Normally present in feces and also found in sewage and water. It is constantly present in milk and is one of the chief causes of the souring of milk and cream.

Morphologically it is variable in length and capsule production. In cultures the growth is abundant and viscid or mucoid in variable degree. It usually ferments the various carbohydrates more vigorously than other colon types, the closed arm of the fermentation tube being usually full or nearly full of gas. Indol production is variable. *B. aërogenes* is much more resistant to acids and other deleterious substances than the other colon types.

**Pathogenesis.**—Probably slight, although it has been isolated from infections of the urinary tract, peritoneum and liver and gall-bladder. In some of the cases reported there is a possibility that the infection was really due to other of the colon types. In the absence of reliable methods of differentiation there is always a question whether the more serious infections are not due to the more virulent bacillus of Friedländer.

**Bacillus Pneumoniae, Friedländer (*B. Mucosus Capsulatus*).**—This bacillus was first described by Friedländer in 1882. He confused this bacillus with the pneumococcus, then undescribed, and regarded it as the causative agent in lobar pneumonia.

**Morphology.**—It varies from coccoid forms to longer bacilli; they may be single, in pairs, or in short chains. They are surrounded by a wide capsule which is easily demonstrable not only in the material directly from the lesions but also in smears from cultures.

**Biology.**—It grows freely on ordinary media. On agar the colonies are characteristically mucoid, with a tendency to confluence. Indol is not produced.

**Pathogenesis.**—It is pathogenic for mice and guinea-pigs in variable degree. Injection intraperitoneally or into the lung is followed by peritonitis or local hepatization with septicemia. Rabbits are killed by some strains after intraperitoneal or intravenous injection; subcutaneous injection, however, commonly causes only a local lesion.

In man it causes both broncho- and lobar pneumonia. This type of pneumonia is relatively infrequent and is characterized by its very high mortality. The bacilli are present in the sputum, as a rule, in almost pure culture. It is commonly present in the upper respiratory tract and the accessory sinuses, and may cause inflammation. By extension, infection of the ear occurs, which may be complicated by meningitis. The pleura or the pericardium may be infected as a complication of pneumonia. A septicemia may develop as a complication of pneumonia or infection elsewhere. Inflammations of the eye are occasionally due to this organism.

**B. Ozena.**—This organism is culturally indistinguishable from the preceding. It receives its name from its constant presence in ozena or fetid rhinitis. Abel and others consider it the causative agent in this disease, basing their opinion on its constant presence.

**Bacillus of Rhinoscleroma.**—This bacillus differs from the *Bacillus pneumoniae* only in its weaker fermentative properties. Its name comes from its frequent presence in rhinoscleroma. Its etiological relationship to this disease is not established. (See Fig. 13.)

**Immunity and Serum Reactions of the Capsulatus Types.**—Active immunity cannot, as a rule, be produced in animals by the injection of killed cultures or their products. Careful injections of animals, however, lead to the production of agglutinins, precipitins, and complement-fixing antibodies. The attempt to differentiate the various types by means of serum reactions has as yet led to no uniform results.

In common with other encapsulated bacteria they are not easily affected by sera. Porges found that the absence of agglutination could be attributed to the presence of the capsule and devised a method of destroying the capsule by first treating the bacterial suspension with acids and heat and then neutralizing with alkali.

**Vaccine Therapy.**—See Part III.

**Other Bacilli Allied to the Colon Group.**—There are various types, of which *B. cloacæ* is an example, which resemble the colon bacillus in many respects but liquefy gelatin in varying degree, some very slightly and only after prolonged growth.

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 MACCONKEY: Jour. Hyg., 1905, v, 333.  
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## CHAPTER XXIII.

### THE TYPHOID BACILLUS.

THIS organism was first observed by Eberth, and independently by Koch, in 1880, in the spleen and diseased areas of the intestine in typhoid cadavers, but was not obtained in pure culture or its principal biological features described until the researches of Gaffky in 1884. The absolute identification of the bacillus only became possible with the increase of our knowledge concerning the specific immune substances. Its etiological relationship to typhoid fever has been particularly difficult to demonstrate, for although pathogenic for many animals when subcutaneously or intravenously inoculated, it was impossible to produce infection in the natural way. More recently infection of the higher apes has been produced. Nevertheless, the specific reactions of the blood serum of typhoid patients, the constant presence of the *Bacillus typhosus* in the intestines and some of the organs of the typhoid cadavers, the very frequent isolation of this bacillus from the roseola, spleen, blood, and excretions of the sick during life, the absence of the bacilli in healthy persons, unless they have at some time been directly exposed to, or are convalescent from, typhoid infection, all these have demonstrated scientifically that this bacillus is the chief etiological factor in the production of the great majority of cases designated as typhoid fever.

**Morphology and Staining.**—Typhoid bacilli are short, rather plump rods of about  $1\mu$  to  $3\mu$  in length by  $0.5\mu$  to  $0.8\mu$  in diameter, having rounded ends, and often growing into long threads. They are longer and somewhat more slender in form than most of the members of the colon group of bacilli (Figs. 129 and 130).

The typhoid bacilli *stain* with the ordinary aniline colors, but a little less intensely than do most other bacteria. Like the bacilli of the colon and paratyphoid groups, they are decolorized by Gram's method. Bipolar staining is sometimes marked.

**Biology.**—The typhoid bacillus is a motile, aërobic, facultative anaërobic, non-liquefying bacillus. It develops best at  $37^{\circ}\text{C}.$ ; above  $40^{\circ}$  and below  $30^{\circ}$  growth is retarded; at  $20^{\circ}$  it is still moderate; below  $10^{\circ}$  it almost ceases. It grows slightly more abundantly in the presence of oxygen. It does not form spores.

**Resistance.**—When a number of typhoid bacilli are dried most of them die within a few hours, the remainder gradually dying during the next few weeks. A few frequently remain alive for months. In their resistance to heat and cold they behave like the average non-spore-bearing bacilli. With rare exceptions they are killed by heating to  $60^{\circ}\text{C}.$  for one minute.

**Motility.**—Typhoid bacilli, when living under favorable conditions, are very actively motile, the smaller ones having often an undulating motion, while the larger rods move about rapidly. In different cultures, however, the degree of motility varies.



FIG. 129.—Typhoid bacilli from nutrient agar.  $\times 1100$  diameters.



FIG. 130.—Typhoid bacilli from nutrient gelatin.  $\times 1100$  diameters.

**Flagella.**—These are often numerous and spring from the sides as well as the ends of the bacilli, but many short rods have but a single terminal flagellum (Figs. 131 and 132; see also Plate III).

**Cultivation.**—Its growth on most sugar-free culture media is quite similar to that of the *Bacillus coli*, but it is somewhat slower and not quite so luxuriant.

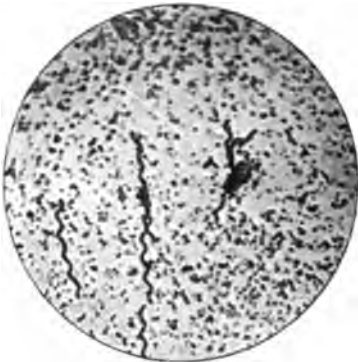


FIG. 131.—Flagella, heavily stained, attached to bacilli. (Van Ermengen's method.)



FIG. 132.—Typhoid bacillus with faintly stained flagella. (Löffler's method.)

**Growth on Gelatin Plates** (Fig. 133).—The colonies growing deep down in this plate medium have nothing in their appearance to distinguish them from submerged colonies of the colon group; they are finely granular, with a sharp margin and a yellowish-brown color. The superficial colonies, however, particularly when young, are often quite characteristic; they are transparent, bluish white in color, with an irregular outline, not unlike a grape leaf in shape. Slightly magnified they



appear homogeneous in structure, but marked by a delicate network of furrows. Surface colonies from some varieties of colon bacilli give a similar picture.

In *stick cultures* in gelatin the growth is mostly on the surface, appearing as a thin, scalloped extension, which gradually reaches out to the sides of the tube. In the track of the needle there is but a limited growth, which may be granular or uniform in structure, and of a yellowish-brown color.

**Growth in Bouillon.**—This medium is uniformly clouded by the typhoid bacillus, but the clouding is not so intense as with the colon bacillus.

When the bouillon is somewhat alkaline a delicate pellicle is sometimes formed on the surface after eighteen to twenty-four hours' growth.

**Growth on Agar.**—The streak cultures on agar are not distinctive; a transparent, filiform, grayish streak is formed.

**Growth on Potato.**—The growth on this medium was formerly of great importance in identification, but now other media, giving more specific characteristics, have been discovered. When characteristic, the growth is almost invisible but luxuriant, usually covering the surface of the medium. Again, the growth may be quite heavy and colored yellowish brown, and with a greenish halo,



FIG. 133.—A superficial colony and a deep colony of typhoid bacilli in gelatin.  $\times 20$  diameters.

when it is very similar to the growth of the colon bacillus. These differences of growth on potato appear to be chiefly due to variations in the substance of the potato, especially in its reaction. For the characteristic growth the potato should be slightly acid. A new lot of potato should always be tested with a typical typhoid bacillus as a control.

**Indol Reaction.**—It does not produce even a trace of indol in peptone-water solution. This test was proposed by Kitasato for differentiating the typhoid bacillus from other similar bacilli such as those of the colon group, which, as a rule, give the indol reaction.

The typhoid bacillus, like the colon bacillus, produces alkaline substances from peptone.

**Neutral Red.**—In stick cultures in glucose agar or glucose broth cultures with neutral red as indicator the typhoid bacillus produces no change, while the colon bacillus decolorizes the medium and produces gas.

**Effect of Inhibiting Substances in Culture Fluids.**—The typhoid bacillus is inhibited by weaker solutions of formaldehyde, carbolic acid, and

other disinfectants than is the colon bacillus. Most typhoid-like bacilli resemble the typhoid bacillus in this respect. Some substances, such as brilliant green, inhibit the colon bacillus more.

**Action on Different Sugars.**—The essential fermentative differences between the typhoid bacillus and paratyphoid colon group are given on p. 324. Acid is produced in mannit, maltose and xylose media. Dulcitol is acted upon, as a rule, after prolonged incubation.

While the typhoid bacillus does not produce gas from dextrose, galactose, and levulose, it does produce acid from these substances.

**Milk.**—The typhoid bacillus does not cause coagulation when grown in milk. A very slight acidity is evidenced in litmus milk from its action on traces of fermentable substances other than lactose.

**Production of Disease in Animals.**—It is impossible, with the exception of the anthropoid apes, to produce a disease like typhoid fever in animals. Metchnikoff and Besredka fed apes with typhoid bacilli by adding fecal material to their food. After eight days' incubation, fever, diarrhea and invasion of the blood stream by the typhoid bacilli, developed. The general clinical picture resembled that of typhoid fever. Some of the animals died. Injection of other animals may produce sickness due to the toxemia produced by the substances in the bodies of the bacilli injected. Typhoid bacilli, freshly obtained from typhoid cases and introduced subcutaneously in animals, rapidly die. In the peritoneal cavity they may increase, causing a fatal peritonitis with toxic poisoning. By accustoming bacilli to the animal body a certain degree of increased virulence for the animal can be obtained, so that smaller amounts of culture may prove fatal. In rabbits a septicemia can be produced by intravenous inoculation. Localization in the gall-bladder follows in some and a "carrier" state similar to that found in man results. Direct injection into the gall-bladder also results in the development of the carrier state.

**Distribution of Bacilli in the Human Subject. Toxic Effects.**—Typhoid fever during its early stages, at least, is accompanied by a bacteriemia. The bacilli thus pass to all parts of the body and become localized in certain tissues, such as the bone marrow, lymphatic tissues and spleen, liver and kidneys. Wherever found in the tissues the typhoid bacilli are usually observed to be arranged in groups or foci; only occasionally are they found singly. These foci are formed during life, as is proved by the degenerative changes often seen about them; but it is possible that the bacilli may also multiply somewhat after death.

**Important Primary Characteristic Lesions in Man.**—The lesions of the intestines which are most pronounced in the lower part of the ileum consist of an inflammatory enlargement of the solitary and agminated lymph nodules. Necrosis with ulceration frequently follows the hyperplasia in the more severe cases. In the severest cases the ulceration and sloughing may involve the muscular and peritoneal coats and perforation may occur. Peritonitis and death usually follow. In rare cases the perforation is closed by adhesions.

The minute changes are a hyperplasia of normal elements of the lymphatic tissue, namely, the lymph cells and the endothelium of the trabeculae and sinuses. In severer forms necrotic changes are apt to intervene. These

changes are attributed to the toxic substances formed by the typhoid bacilli, but may be directly brought about by the occlusion of the nutritive blood-vessels, as pointed out by Mallory.

The mesenteric lymph nodes undergo changes similar to those in the ileum. The spleen is enlarged because of congestion and hyperplasia. The liver and, to a less extent, the kidneys are apt to show foci of cell proliferation.

In typhoid fever, as in other infectious diseases, toxic poisoning may be manifested by disturbances in the circulatory, respiratory, and heat-regulating mechanism as well as by manifest lesions. In a few cases the intestinal lesions are absent. Some of the inflammatory complications which occur in typhoid fever are due to the growth of the bacillus in excessive numbers in unusual places in the body; but many of them are due to a secondary infection with other bacteria, especially the pyogenic cocci and bacilli of the colon group.

**Unusual Location of Typhoid Lesions Occurring as Complications of Typhoid Fever.**—Cases of sacculated and general peritonitis, abscess of the liver and spleen, subphrenic abscess, osteomyelitis, periostitis, and inflammatory processes of other kinds have been reported as being due to the typhoid bacillus. In certain cases of typhoid pneumonia, serous pleurisy, empyema, and inflammations of the brain and spinal cord or their membranes, typhoid bacilli exclusively have occurred. The inflammation produced may or may not be accompanied by the formation of pus. There are indeed a number of cases now on record in which the typhoid bacillus has played the part of *pus producer*.

**The Importance of Mixed Infection.**—Frequently when complications occur in typhoid fever they are due to secondary or *mixed infection* with the staphylococcus, pneumococcus, streptococcus, pyocyanus, and colon bacillus. Frequently these bacteria are found side by side with typhoid bacilli; in such cases it is difficult to say which was the primary and which was the secondary infection.

**Primary Infection of Liver and Gall-bladder.**—We find an appreciable number of chronic typhoid carriers with no history of having had typhoid. Although the symptoms may have been so slight as to have been overlooked, it is probable that bacilli may escape from the intestine into the portal circulation and thus infect the liver and gall-bladder (see Normal Carriers).

**Elimination of Typhoid Bacilli from Body.**—Not infrequently typhoid bacilli are found in the secretions. They are present in the urine in about 25 to 50 per cent. of the cases during some part of the disease. Slight pathological lesions in the kidneys almost always occur in typhoid fever, but severe lesions also sometimes occur. In some cases the urine has very many typhoid bacilli.

In cases of pneumonia due to the typhoid bacillus it is abundantly present in the sputum, and care should be taken to disinfect the expectoration of typhoid patients. In typhoid fever the bacilli are almost always present in the gall-bladder. The bacilli are usually eliminated by the feces, being derived from the ulcerated portions of the intestines; their growth within the intestinal contents is, with few exceptions, not extensive. They may be excreted, however, very early in the disease, and have been found in the stools even during the incubation period.

Not only the great majority of cases examined bacteriologically and pathologically, but the epidemiological history of the disease, proves that the chief mode of invasion of the typhoid bacillus is by way of the mouth and stomach. The infective material is discharged principally by means of the excretions and secretions of the sick—namely, by the feces, the urine, and occasionally by the sputum.

**Typhoid Carriers.**—The bacilli usually disappear from the body during the first week or two of convalescence. About 1 to 5 per cent. continue to excrete typhoid bacilli for years, perhaps for life. Petruschky, in 1898, reported that typhoid bacilli sometimes remained in the urine of typhoid convalescents for months. Cushing soon after observed a case who had had typhoid fever five years before. In 1902 Frosch, and a little later Conradi and Drigalski, reported persons who passed typhoid-infected feces months after recovery from typhoid fever. Some bacilli carriers did not know either that they had had typhoid fever or been in contact with it, and others knew only that they had been in contact with it.

In Washington, during the typhoid season, a series of one thousand examinations showed that 0.3 per cent. of persons with no known exposure, and who had not had typhoid were excreting typhoid bacilli. These cases may be called "healthy or normal carriers" in contradistinction to "convalescent carriers." Lentz, in 1905, found out of a large number of examinations that about 4 per cent. of persons convalescent from typhoid fever were typhoid carriers. In our laboratory we have found six in one hundred and forty institution convalescents. The focus of infection is either the gall-bladder or bile ducts. The majority are women.

A remarkable case of a cook ("Typhoid Mary"), discovered by Soper, was under our care for three years. A visitor of the family in which this woman was cook developed typhoid fever some ten days after entering the household. This was in 1901. The cook had been with the family three years and it is difficult to judge which infected the other. The cook went to another family. One month later the laundress in this family was taken ill.

In 1902 the cook obtained a new place. Two weeks after arrival the laundress was taken ill with typhoid fever; in a week a second case developed and soon seven members of the household were sick.

In 1904 the cook went to a home in Long Island. There were 4 in the family as well as 7 servants. Within three weeks after arrival 4 servants were attacked.

In 1906 the cook went to another family. Between August 27 and September 3, 6 out of its 11 inmates were attacked with typhoid. At this time the cook was first suspected. She entered another family on September 21. On October 5 the laundress developed typhoid fever.

In 1907 she entered a family in New York City, and two months after her arrival 2 cases developed, 1 of which proved fatal.

The cook was removed to the hospital March 19, 1907. Cultures taken every few days showed bacilli off and on for three years. Sometimes the stools contained enormous numbers of typhoid bacilli and

again for days none would be found. She was released on parole in 1910, promising to report to the Health Department and not to engage in cooking. She broke her parole and disappeared. In 1915 in an epidemic of typhoid at a maternity hospital, a total of 25 cases developed. Investigation showed that food infection was the cause and the cook was identified as "Typhoid Mary." During the period of disappearance she infected a friend and was the cause of several cases in a small private sanatorium. She is known to have been the cause of at least 50 cases of typhoid fever. We recently traced some hundreds of cases of typhoid fever to a milk supply produced at a farm, looked after by a typhoid carrier who had typhoid fever forty-seven years ago.

**Treatment of Typhoid Carriers.**—Medicinal treatment or surgery seems so far to have yielded only slight results. Urotropin in very large amounts is reported to have cured one case, in which operation alone had failed. Removal of the gall-bladder cannot be relied upon, as the bacilli may be present in the bile ducts. Meader reports some success with long-continued treatment by injections of killed cultures.

**Duration of Life in Water, Feces, Oysters, etc.**—It is of importance to know for what length of time the typhoid bacillus is capable of living outside of the body; but, unfortunately, owing to the great difficulties in proving the presence of this organism in natural conditions, our knowledge on this point is still incomplete. In feces, in privies, on the ground, etc., the length of life of the typhoid bacilli is very variable, depending on the composition of the feces and the soil, and on the varieties of bacteria present; sometimes they live but a few hours, usually a day, exceptionally for very long periods. Thus, according to Levy and Kayser, in winter typhoid bacilli may remain alive in feces for five months. Foote says that they can be found in living oysters for a month at a time, but in numerous experiments we have not been able to find them after five days. Their life in privies and in water is usually short, often not over forty-eight hours and usually not over a week. The less the general contamination of the water, the longer the bacilli are apt to live. The life of the typhoid bacillus varies according to the abundance and varieties of the bacteria associated with it, and according to the presence or absence of such injurious influences as deleterious chemicals, high temperature, light, etc., to which it is known to be sensitive. In ice typhoid bacilli rapidly die, none probably ever live as long as six months (see p. 348).

**Methods of Communication.**—The bacilli may reach the mouth by means of infected fingers or articles of various kinds, or by the ingestion of infected food, milk, water, etc., or more obscure ways, such as the eating of raw oysters and clams or the contamination of food by flies. Of great importance, though gradually lessening, however, is the production of infection by contaminated drinking water or milk. In a very large number of cases indirect proof of this mode of infection has been afforded by finding that the water had been contaminated with urine or feces from a case of typhoid. In a very few instances the proof has been direct—namely, by finding typhoid bacilli in the water.

Examples of infection from water and milk have frequently come under our direct observation. The following instances may be cited: A large force of workmen obtained their drinking water from a well near where they were working. Typhoid fever broke out and continued to spread until the well was filled up. Investigation showed that some of the sick, in the early stages of their disease, repeatedly infected the soil with their urine and feces at a point which drained into the well. Another example occurred in which typhoid fever broke out along the course of a creek after a spring freshet. It was found that, far up near the source of the creek, typhoid feces had been thrown on one of its banks and had then been washed into the stream.

The epidemic at Scranton, Pa., during the winter of 1907 was most interesting. A little over 1 per cent. of the inhabitants were attacked. No pollution of the water with typhoid feces or urine could be discovered, although typhoid bacilli were isolated from the water of a small intercepting reservoir by Dr. Fox. This was only accomplished by using large quantities of water. The bacillus isolated was identical by all known tests with the typhoid cultures from cases of typhoid fever. A stream entering the reservoir was exposed to pollution by men working on a nearby railroad.

In water-borne outbreaks of typhoid fever it is not infrequent to have outbreaks of diarrhea preceding or accompanying the development of the typhoid fever. The number of cases of diarrhea may be many times greater than the number of typhoid cases. A sudden outbreak of diarrhea therefore may mean contamination of a water supply and should be considered as possible indication of an oncoming typhoid outbreak.

An instance of milk infection secondary to water infection occurred in the case of a milk dealer whose son came home suffering from typhoid fever. The feces were thrown into a small stream which ran into a pond in which the milk cans were washed. A very alarming epidemic of typhoid developed, which was confined to the houses and asylums supplied with this milk. Milk-borne epidemics are most commonly due to infection of the milk by carriers. During the Spanish-American War not only water infection, but food infection was noticed, as in the case of a regiment in which certain companies were badly infected, while others nearly escaped. Each company had its separate kitchen and food supply, and much of the infection could be traced to the food, the contamination coming partly through the flies. A small number of cases have been traced to oysters. *Contact infection* is responsible for many cases. Even during outbreaks due to various causes, contact infection plays a large role in the development of secondary cases. These cases develop either by contact with known typhoid cases or through convalescent or healthy carriers in the households. The early undiagnosed cases may have typhoid bacilli in their stools and act as a source of contagion; likewise short, mild cases develop which are never diagnosed. For these reasons "typhoid precautions" should not await positive diagnosis, but should be instituted on the slightest suspicion.

**Individual Susceptibility.**—In this, as in all infectious diseases, *individual susceptibility* plays an important role in the production of infection. Where exposure is associated with the ingestion of few bacilli, the majority of persons escape infection. In many individuals there probably is some disturbance of the digestion, excesses in drinking, etc., or a general weakening of the power of resistance of the individual, caused by bad food, exposure to heat, overexertion, etc., as occurs with soldiers and prisoners, for example, to bring about the conditions suitable for the production of typhoid fever, when the dose of bacilli is small. Ingestion of larger numbers of bacilli and especially with repeated exposure, 50 per cent. or more of the exposed succumb to infection.

**Immunization.**—After recovery from typhoid fever a considerable immunity is present which lasts for years. This is not absolute, as about 2 per cent. of those having typhoid fever have a second attack, which is usually a mild one. Specific *immunization* against experimental typhoid infection has been produced in animals by the usual method of injecting at first small quantities of the living or dead typhoid bacilli and gradually increasing the dose. The blood serum of animals thus immunized has been found to be highly bactericidal and to possess some protective power against toxic extracts of the bacilli. It is also rich in agglutinins, precipitins, and opsonins. These characteristics have also been observed in the blood serum of persons who are convalescent from typhoid fever. The attempt has been made to employ the typhoid serum for the cure of typhoid fever in man, but, although a number of individual observers have reported good results with one or another of the serums, most consider that little or no good is derived from serum.

**Vaccine and Serum.**—See Part III.

**Diagnosis by Means of the Widal or Agglutination Reaction.**—The chief practical application of our knowledge of the specific substances developed in the blood of persons sick with typhoid fever has been as an aid to diagnosis.

**Gruber-Widal Test.**—The first application of the use of serum for the early diagnosis of typhoid fever on an extensive scale was made by Widal, and reported with great fulness and detail in a communication published in June, 1896. For history and theory of test see p. 179. Since then the serum test for the diagnosis of typhoid fever has come into general use in bacteriological laboratories in all parts of the world, and though the extravagant expectations raised at the time when Widal first announced his method of applying this test have not been entirely fulfilled, it has, nevertheless, proved to be of great assistance in the diagnosis of obscure cases of the disease, and is now one of the recognized tests for the differentiation of the typhoid bacillus.

It should also be mentioned that to Wyatt Johnson, of Montreal, belongs the credit of introducing its use into municipal laboratories, suggesting that dried blood should be employed in place of blood serum.

**Use of Dried Blood.**—**Directions for Preparing Specimens of Blood.**—The skin covering the tip of the finger or the ear is thoroughly cleansed,

and is then pricked with a needle deeply enough to cause several drops of blood to exude. Two fair-sized drops are then placed on a glass slide, one near either end, and allowed to dry. Glazed paper may also be employed, but it is not as good, for the blood soaks more or less into it, and later, when it is dissolved, some of the paper fiber is apt to be rubbed off with it. The slide is placed in a box for protection.

**Preparation of Specimen of Blood for Examination.**—In preparing the specimens for examination, the dried blood, if accuracy is desired, is first weighed and then brought into solution by adding to it the quantity of normal salt solution to make the desired dilution, remembering of course to allow for the loss of the water in the blood through drying. The loss of water is equivalent to about 80 per cent. of the weight of the blood. A drop of each dilution is placed on individual cover-slips and to each is added a drop of an eighteen-hour broth culture of typhoid. These are then mounted on hollow slides in the ordinary way. (See p. 71.) The drops, after being mixed, have in a 1 to 10 dilution a distinct reddish color and in 1 to 20 a faint pink tinge. The cover-glass with the mixture on the surface is inverted over a hollow slide (the edges about the concavity having been carefully smeared with vaselin, so as to make a closed chamber). Ordinarily the dried blood is not weighed, but the measure of dilution is estimated by the color of the drop. To judge this the beginner must carefully make dilutions of fluid blood and notice the depth of color in 1 to 10 and 1 to 20 dilutions. Besides the faulty judgment of the dilution color by the examiner, the variation in depth of color of different specimens of blood makes the estimation of dilutions more or less inaccurate, but fortunately this does not greatly interfere with the value of the test.

For use of serum or fluid blood see Agglutination for methods, p. 202.

**Advantages and Disadvantages of Serum, Dried Blood, and Fluid Blood for the Serum Test.**—The dried blood is easily and quickly obtained, and does not deteriorate or become contaminated by bacterial growth. It is readily transported, and seems to be of nearly equal strength as the serum in its agglutinating properties. It must in use, however, be diluted with at least five times its original bulk with water, otherwise it is too viscid to be properly employed. The amount of dilution can be determined roughly by the color of the resulting mixture. Serum, on the other hand, can be used in any dilution desired, from a mixture which contains equal parts of serum and broth culture to any dilution desired; and this can be exactly measured by a graduated pipette, or, roughly, by a measured platinum loop. The disadvantages in the use of serum are entirely due to the slight difficulty in collecting and transporting it. If the serum is obtained from blood after clotting has occurred a greater quantity of blood must be drawn than is necessary when the dried-blood method is used. For scientific investigations and for accurate results, particularly in obscure cases, the use of serum is to be preferred to dried blood. Practically, however, the results are nearly as good for diagnostic purposes from the dried blood as from the serum.



**The Culture to be Employed.**—It is important that the culture employed for serum tests should be a suitable one, for although all cultures show the reaction, yet some respond much better and in higher dilutions than others. Cultures freshly obtained from typhoid cases are not as sensitive as those grown for some time on nutrient media. Those kept for a long time on artificial media sometimes show a decided tendency to spontaneous agglutination. Artificial cultivation is usually accompanied by increase of capacity for agglutination. At present a strain known as Mt. Sinai is used at this laboratory. A broth culture of the typhoid bacillus developed at 25° to 35° C., not over twenty-four hours old, in which the bacilli are separated and actively motile, has been found to give us the most satisfactory results. If the broth culture is heavy it should be diluted. Cultures grown at temperatures over 38° C. are not apt to agglutinate so well as those grown at lower temperatures.

**Dilution of the Blood Serum to be Employed and Time Required for the Development of Reaction.**—The serum test, as has been pointed out, is quantitative and not qualitative, that is, the diagnostic reaction must be in dilutions higher than occur because of the presence of normal or group agglutinins. (See p. 204.) It is most important to remember that it is purely a matter of experience to determine in any type of infection what agglutinating strength of a serum is of diagnostic value.

The results obtained in the Health Department Laboratories, as well as elsewhere, have shown that in a certain proportion of cases not typhoid fever a slow reaction occurs in a 1 to 10 dilution of serum or blood; but very rarely does a complete reaction occur in this dilution within *fifteen minutes*. When dried blood is used the slight tendency of non-typhoid blood in 1 to 10 dilution to produce agglutination is increased by the presence of the fibrinous clumps, and perhaps by other substances derived from the disintegrated blood cells.

From many cases examined it has been found that in dilutions of 1 to 20 a quick reaction is almost never produced in any febrile disease other than that due to typhoid or paratyphoid bacillus infection. In typhoid fever such a distinct reaction often occurs with dilutions of 1 to 100 or more. It is possible that some cases of paratyphoid infection give a prompt reaction in 1 to 20 dilutions, but if this is so, it is not a serious drawback. Typhoid-bacillus carriers with the exception of transient contact or normal carriers, commonly have specific agglutinins in their blood whether they previously have had typhoid fever or not.

The mode of procedure as now employed is as follows: With serum, one part of a 1 to 10 dilution is added to one of the bouillon cultures. With dried blood, as stated above, a solution of the blood is first made according to a known color, and the dilution is made from this. If there is no reaction—that is to say, if within five minutes no marked change is noted in the motility of the bacilli, and no clumping occurs—nothing more is needed; the result is negative. If marked clumping and immobilization of the bacilli immediately begin and become complete within five minutes, this is termed a *marked immediate typhoid reaction*, and no further test is considered necessary, though it is always advisable to confirm the reaction with higher dilutions up to 50 or

more, so as to measure the exact strength of the reaction. If in the 1 to 20 dilution a complete reaction takes place within thirty minutes, the blood is considered to have come from a case of typhoid infection, while if a less complete reaction occurs it is considered that only a probability of typhoid infection has been established. By many the time allowed for the development of the reaction with the high dilutions is from one to two hours, but to us twenty minutes with the comparatively low dilution of 1 to 20 seems safer and more convenient. Positive results obtained in this way may be considered conclusive, unless there be grounds for suspecting that the reaction may be due to a previous fairly recent attack or to recent vaccination. The failure of the reaction in one examination by no means excludes the presence of typhoid infection. If the case clinically remains doubtful, the examination should be repeated every few days.

**Use of Dead Cultures.**—Properly killed typhoid bacilli respond well to the agglutination test. For the physician at his office the dead bacilli offer many advantages. The reaction is slower than with the living cultures and is observed either macroscopically or microscopically. A number of firms now supply outfits for the serum test. These outfits consist of a number of small tubes containing an emulsion of dead typhoid bacilli. Directions accompany the outfit. This method has been found of great value in comparative estimation of agglutinin by Dreyer and others.

**Proportion of Cases of Typhoid Fever in which a Definite Reaction Occurs, and the Time of its Appearance.**—As the result of a large number of cases examined in the Health Department Laboratories, it was found that about 20 per cent. give positive results in the first week, 60 per cent. in the second week, about 80 per cent. in the third week, about 90 per cent. in the fourth week, and about 75 per cent. in the second month of the disease. In 98 per cent. of the cases in which repeated examinations were made (hospital cases) a definite typhoid reaction was present at some time during the illness.

**Persistence of the Reaction after Convalescence or after Immunization.**—A definite typhoid reaction has been observed from three months to a year after convalescence, and a slight reaction, though much less than sufficient to establish a diagnosis of typhoid infection, from one to fifteen years after the disease. In persons in whom the typhoid bacilli persist the serum reaction may last as long as the bacilli remain in the body. As a rule the reaction becomes progressively weaker during the first six months after vaccination, and has usually completely disappeared at the end of a year.

**Typhoid Bacilli in the Blood.**—A blood culture is usually positive during the first week of typhoid fever, and is the best method for early diagnosis. Bacilli appear in the blood even in the first few days of the disease. In the first week, nearly 100 per cent. positive results are obtained, in the second week 50 per cent. and then progressively less till the end of the disease. In relapses they reappear.

**Method.**—The blood is drawn from the median basilic vein by syringe and is inoculated into either broth or bile medium. If broth is used,

several flasks are used with sufficient broth to dilute the blood 50 times or more. The essential thing is to dilute the blood sufficiently so that coagulation does not occur. The separated serum apparently is more strongly bactericidal and inhibits growth.

Bile mediums are more convenient, as the bulk need not be so large. The bile inhibits coagulation. Three parts of bile medium are used to one part of blood. The bile is plated on Endo, or Conradi, after eighteen hours' incubation. It should also be examined after two or three days, as growth is sometimes delayed.

Another method, not as satisfactory as the preceding, but valuable in emergencies, is to inoculate the blood into ten times its volume of distilled water. Hemolysis results and the proteins supply the nutrient substances for growth.

**Typhoid Bacilli in Feces.**—The following table by Schröder gives the number and time of positive results:

	No. examined.	Positive.	Per cent.
First week . . . . .	115	11	9.6
Second week . . . . .	160	45	28.1
Third week . . . . .	71	16	22.5
Fourth week . . . . .	41	9	21.9
Fifth week . . . . .	26	4	15.3
Sixth week . . . . .	12	2	16.6
Convalescent . . . . .	190	10	7.9

These results were obtained using several media in combination.

The more promptly the stools are plated after passage, the better is the chance of obtaining typhoid bacilli. Hiss, examining the stools very quickly after passage, obtained a very high percentage of positive results, in 43 consecutive cases, 37 of which were in the febrile stage and 6 convalescent. In a number of instances only one stool was examined, but even under these adverse conditions the average of positive results in the febrile stage was 66.6 per cent. Out of 26 cases of typhoid fever examined in hospitals, 21 were in the febrile stage and 5 convalescent. In 17 of the febrile cases the presence of typhoid bacilli, often in great numbers, was demonstrated. Thus in these carefully followed cases the statistics show over 80 per cent. of the febrile cases positive. The bacilli were isolated from these cases as early as the sixth day, and as late as the thirtieth day, and in a case of relapse on the forty-seventh day of the disease. These observations, with regard to the appearance of the bacilli in the stools during the febrile stage and their usually quick disappearance, except in the permanent typhoid carriers, after the defervescence, have been confirmed by others. We have, however, a carrier under observation who was discharged from the hospital after three negative examinations. The bacilli are isolated in some cases before the appearance of the Widal reaction; and in some cases in which no Widal reaction was demonstrated. Between the seventh and twenty-first day of the disease, experience seems to indicate that the bacilli may be obtained from about 25 per cent. of all cases on the first examination and from about 75 per cent. after repeated examinations. In some samples of feces typhoid bacilli die out or are overgrown within twenty-four hours; in others they remain alive for days or even weeks. This

seems to depend on the bacteria present in the feces and upon its chemical character. Probably the presence of typhoid bacilli in some stools and their absence in others must be explained largely by the characteristics of the intestinal contents. The short life of the typhoid bacillus in many specimens of feces suggests that stools be examined as quickly as possible. In fact, unless the physician wishes to take the trouble to have the sample of feces sent immediately to the laboratory, it is hardly worth while for the bacteriologist to take the trouble to make the test. (When specimens are delayed in transit the method suggested by Teague and Clurman can be employed. One part of feces should be added if fluid, or well rubbed up if solid, in two parts of 30 per cent. glycerin in 0.6 per cent. salt solution. This concentration of glycerin has no effect on the typhoid bacillus but prevents its overgrowth by the fecal bacteria.)

**Method.**—The feces, if solid, are rubbed up with peptone-water, otherwise they can be used without further preparation. The fluidified feces should consist of a mixture of a generous amount of the whole mass, not merely a loopful.

The density of the suspension to employ is a matter of experience or successive dilutions may be plated. For general use the Endo or Conradi medium are most frequently employed. We employ Endo and a brilliant green agar, using two concentrations of brilliant green (see p. 107) which by suppression of some or all of the fecal flora allows a heavy inoculation with coincident increase in positive results. None of the fluid enrichment media that we have tried have been satisfactory for general use, although they may be successful in isolated cases. We have had no experience with the recently published method of Teague and Clurman which may yield better results. Plates are streaked (see p. 108) and the colonies of typhoid can then be agglutinated directly either microscopically or by using the slide method. In the latter a drop of saline for control and a drop of high-titre serum are placed on a slide and the colony rubbed up in the saline and then in the serum. With appropriate dilutions of the serum, agglutination will take place almost immediately.

For fishing, Russell's medium is used, which gives a tentative indication of the nature of the organism, which can be confirmed by agglutination and further cultural tests.

**Typhoid Bacilli in the Urine.**—Of great interest is the frequent occurrence of typhoid bacilli in large numbers in the urine. The results of the examinations of others as well as our own indicate that the typhoid bacilli are not apt to be found in the urine until the end of the second week of the fever, and may not appear until much later. From this on to convalescence they appear in about 25 to 50 per cent. of the cases, usually in pure culture and in enormous numbers, even as high as 100 million per cubic centimeter. They are found until several weeks or months after convalescence; in exceptional cases they persist for years. When we think of the chances such cases have to spread infection as they pass from place to place, we begin to realize how epidemics can start without apparent cause. The more we investigate the per-

sistence of bacteria in convalescent cases of disease, the more difficult the prevention of their dissemination is seen to be. The disinfection of the urine should always be looked after in typhoid fever, and convalescents should not be allowed to go to places where contamination of the water supply is possible, without at least warning them of the necessity of great care in disinfecting their urine and feces for some weeks.

**Method.**—If the bacilli are numerous, as evidenced by examination in the hanging drop, they are easily obtained by direct plating, otherwise the urine should be centrifuged and the sediment plated, and larger amounts inoculated into bile medium for enrichment.

**Typhoid Bacilli in Rose Spots and Spleen.**—Although the bacilli have been frequently isolated from rose spots, it is a less convenient method than blood cultures. Spleen puncture has been employed and although cultures are usually positive the operation is dangerous, and has been abandoned.

**Detection of Typhoid Bacilli in Water.**—There is absolutely no doubt that the contamination of streams and reservoirs is a frequent cause of the outbreak of epidemics of typhoid fever, but the actual finding and isolation of the bacilli is a very rare occurrence. This is often due to the fact that the contamination has passed away before the bacteriological examination is undertaken, and also to the great difficulties encountered in detecting a few typhoid bacilli when they are associated with large numbers of other bacteria.

**The Importance of Ice in the Production of Typhoid Fever.**—The total number of instances of typhoid fever which have been reasonably attributed to ice infection are remarkably few. One was in France, where a group of officers placed ice made from water polluted by a sewer in their wine and afterward a large percentage developed typhoid fever, while those of the same company not using ice escaped. A second instance was a small epidemic which occurred among those who used ice from a pond. It was found that water directly infected with typhoid feces had flowed over its frozen surface and been congealed there.

The fact that freezing kills a large percentage of typhoid bacilli makes it indeed possible to conceive that ice from moderately infected water while still polluted contains so few living typhoid bacilli that only the exceptional person here and there becomes infected, and so the source of the infection remains undetected.

If this be true and scattered cases occur, there should be at least some increase in some if not every year in March, April, and May in such a city as New York, where four-fifths of all the ice consumed is from the Hudson River, which is known to be contaminated with typhoid bacilli. Many persons place the ice directly in their drinking water.

When we examine the records for the past ten years we find no increase of typhoid fever in Greater New York during those months, with the one exception of 1907, when we had in the borough of Manhattan a sharp outbreak lasting four weeks. This outbreak did not occur at all in Brooklyn. As the people of Brooklyn drank different

water, but received ice from the same places of the Hudson River as those of Manhattan, this directed attention to the water or milk rather than the ice. Examination of the Croton watershed at the time showed that a small epidemic of typhoid existed there and that pollution of the water was probable. This suggested still more strongly that the water and not the ice was the cause of the typhoid infection.

It happened that most of the cases occurred in those living in the section of the upper West Side, where only well-to-do people live. An investigation showed that the majority of the infected had used only artificial ice and several had used no ice in their water at all.

**Life of the Typhoid Bacillus in Ice in Laboratory Experiments.**—The first important investigation was that of Prudden, who showed that typhoid bacilli might live for three months or longer in ice. This experiment is frequently wrongly interpreted, as when a recent writer states: "It has been amply demonstrated that the germs of typhoid fever are not killed by freezing and that they have been known to live in ice for long periods of time."

It is true that in Prudden's experiment a few typhoid bacilli remained alive for three months, when the experiment was terminated, but those were but a small fraction of 1 per cent. of the original number. Following Prudden's experiment Sedgwick and Winslow, in Boston, and Park, in New York City, carried on independently a series of experiments. These led to the same conclusions. A table summarizing a final experiment of ours in which twenty-one different strains, mostly of recent isolation, were subjected to the test is given below:

LIFE OF TWENTY-ONE STRAINS OF TYPHOID BACILLI IN ICE.

	Average number of bacilli in 1 gm. of ice.	Percentage typhoid bacilli living.
Before freezing . . . . .	2,560,410	100.0
Frozen three days . . . . .	1,089,470	42.0
Frozen seven days . . . . .	361,136	14.0
Frozen fourteen days . . . . .	203,300	8.0
Frozen twenty-one days . . . . .	10,280	0.4
Frozen five weeks . . . . .	2,950	0.1
Frozen nine weeks . . . . .	127	0.005
Frozen sixteen weeks . . . . .	107	0.004
Frozen twenty-two weeks . . . . .	0	0

In these experiments twenty-one different flasks of Croton water were inoculated each with a different strain of typhoid bacilli. In one a little of the feces rich in typhoid was directly added. The infected water in each flask was then pipetted into thirty tubes. These tubes were placed in a cold-storage room in which the temperature varied from 20° to 28° F. At first tubes were removed and tested twice a week, later once a week. The object of using so many different strains was because it has become evident that some cultures live longer than others.

At the end of five weeks the water infected with six cultures was sterile, at the end of sixteen weeks only four strains remained alive.

Interesting investigations of Hudson River ice were carried out in 1907 by North.

There was noticed a considerable difference between the number of bacteria in the top, middle, and bottom layers of ice. This is natural, since while water in freezing from above downward markedly purifies itself, 75 per cent. of the solids and a fair proportion of bacteria being eliminated, yet this cannot happen in the case of the snow blanket which becomes flooded by rain or by cutting holes through the ice. Here all impurities, such as dust and leaves which have fallen on the surface and dirt which may come from the water, remain with the bacteria which they carry, since all are retained in the porous snow. The bacteria in freshly cut bottom ice generally show the least destruction by freezing.

The river water in the forty specimens averaged 1800 bacteria per cubic centimeter, the top ice 306, the bottom ice 36, and the middle ice 14. Only four specimens of top ice had over 500 bacteria per cubic centimeter; none of the specimens of middle or bottom ice.

The great destruction by freezing is noticeable in these figures. Even the top ice soiled by flooding and by the horses and men gathering it contained but 16 per cent. as many bacteria as the water from which it was obtained. The bottom ice, the last to be frozen, had but 2 per cent. of those in the water.

**Conclusions in Regard to Ice Pollution.**—The danger from the use of ice produced from polluted water is always much less than the use of the water itself. Every week that the ice is stored the danger becomes less, so that at four weeks it has become as much purified from typhoid bacilli as if subjected to sand filtration. At the end of four months the danger becomes almost negligible, and at the end of six months quite so.

**Differential Diagnosis.**—The typhoid bacillus is easily separated from the other members of the group, and for practical purposes it is sufficient identification if the colony on the special plating media and the growth on Russell's medium is characteristic and is agglutinated in relatively high dilutions of a serum, so as to eliminate the action of group agglutinins. If a strain does not agglutinate freely, as commonly happens with strains isolated from the blood, it should be transferred daily on plain agar and again tested. The range of group agglutinins in the serum used should be known or error will result.

In important examinations or strains from unusual sources as water, etc., a more extensive cultural study should be done. Likewise, absorption of agglutinins and possibly the production of agglutinins for a known strain should be tested.

**B. (fecalis) Alkaligenes.**—This group resembles the typhoid bacillus but produces acid from no sugars. It is frequently present in the intestines. It is usually only a saprophyte, but has been found in a few cases of disease in man.

Other non-gas-producing typhoid-like bacilli have been isolated not only from the feces of man but also from feces of cholera-infected swine, cow's feces, and water.

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## CHAPTER XXIV.

### PARATYPHOID GROUP.

GÄRTNER, in 1888, found the *Bacillus enteritidis* in association with a meat-poisoning epidemic. A cow sick for two days with profuse diarrhea had been slaughtered and the meat sold for food. Of the persons eating the meat fifty-seven became ill and one died.

Similar bacilli were isolated by Smith from swine suffering from hog cholera, from mice by Löffler, and the bacilli became known as the hog cholera group.

Similar bacilli were isolated by others from cases resembling typhoid fever. Schottmüller reported in 1900 that he was able to differentiate the strains from 5 cases into two groups, one resembling typhoid very closely, the other corresponding to the enteritidis types. The work of Durham and Buxton gave the basis for classification into subgroups.

Numerous types have been isolated from infections in man and animals, and most of the terms in use refer to their source; but many cannot be differentiated one from the other, so fall into distinct groups according to their bacteriological and immune reactions. Gas production from glucose is usually given as the differential characteristic between this group and *B. typhosus*. This is true in general for the human pathogens. One type, *B. sanguinarium* from fowls, produces no gas. This characteristic is susceptible to variation and may be lost. From our own work it would appear that the ability to ferment rhamnose is the basic characteristic of the paratyphoid enteritidis group. The more the strain resembles the typhoid bacillus, however (especially the non-gas-producing type mentioned above), the lower is the avidity for this carbohydrate. The ability to ferment salicin or saccharose, serves to exclude many of the paratyphoid-like organisms found in feces. Indol also is not produced by the types known to be pathogenic for man.

**Paratyphoid A.—Disease Produced.**—First isolated by Gwyn, later by Schottmüller, Brown, and Kayser, and others. It produces a typhoid-like disease in man, but is of relatively infrequent occurrence. The bacillus may be present in the feces, urine, blood, and bile. The post-mortem findings have been variable. In a few the typical intestinal lesions of typhoid infections were present, in others they were absent. A diffuse inflammation of the intestines may be found.

**Morphology and Biology.**—Similar to typhoid in many respects. Krumwiede has recently shown that it differs from all other members of the paratyphoid-enteritidis group in that it does not produce acid from xylose. Not all the non-xylose-fermenting types from man are alike agglutinatively and he suggests that all be included in the paratyphoid "A" group and that the strains differing agglutinatively be



considered a subgroup. This fermentative reaction is more reliable than the differentiation by litmus milk. The reaction on litmus milk has, since Schottmüller's work, been relied upon for differentiation. The reaction, however, is only quantitative and exceptions occur.

**Communicability.**—Communicability is the same as for typhoid. It has been isolated in a few instances from animals, and perhaps in water and foodstuffs, but its distribution can in no way be compared with that of the other members of this group.

**Diagnosis.**—See below.

**Paratyphoid B.**—This group includes many types, isolated not only from disease of man but also from diseases of many animals.

**Disease Produced in Man.**—This varies and may be subdivided into several clinical types, depending on whether the disease is primarily an infection or an intoxication.

**Typhoid-like Type.**—A variable period of incubation is usually present. The onset is commonly more acute than in typhoid fever, and is usually accompanied by chills. As a rule it runs a shorter and milder course. In some epidemics abortive attacks occur with no symptoms except fever for a short time. The bacilli may be present in the feces, blood, urine, and gall-bladder; in the first two, early in the disease and commonly during the whole course. Complications similar to those in typhoid may occur.

**Gastro-enteric Type.**—The onset is acute and the symptoms come on promptly after eating the infected food or after twenty-four to forty-eight hours. The symptoms are those of an acute enteritis ushered in by chills and rise of temperature. Nervous symptoms are common and may be severe. The bacillus can be isolated from the blood as early as the first day of the disease. In the mild cases cultures are negative. They are present in the stools during the acute stage but quickly disappear in the acute cases.

**Cholera-like Types.**—These differ from the preceding only in the degree of the toxic and intestinal symptoms. The bacilli may be present in the blood.

Localized infections occur as complications, occasionally as primary infections without any other symptoms of paratyphoid infection elsewhere, such as pyelitis (sometimes associated with septicemia), cystitis, arthritis, periostitis, phlebitis, cholecystitis, etc. Infection with these types occasionally occurs as a complicating infection of other diseases.

**Morphology and Biology.**—They closely resemble the other members of this group. For differential characteristics see pp. 324 and 351.

**Occurrence in Healthy Persons; Paratyphoid Bacillus Carriers ("A" and "B" Types).**—When general invasion occurs the bacilli may be found in the urine after convalescence for shorter or longer periods of time. Its localization in the gall-bladder and its excretion in the feces by chronic carriers is fairly frequent. We recently had the opportunity of examining the feces of every man in a militia regiment which had been badly infected with paratyphoid "A", while at the Mexican border. We found nearly 4 per cent. of healthy carriers. This incidence of

normal carriers is interesting in relation to normal carriers of *B. typhosus*. Is this greater incidence only apparent, due to the greater ease with which we can isolate *B. paratyphosus* as compared with *B. typhosus* or actual, because the former known to be less virulent, a greater number of persons escape infection but become carriers for a shorter or longer time? Careful bacteriological examination has revealed paratyphoid-like bacilli in the feces of a considerable percentage of healthy persons. This fact is unfortunately lost sight of, and etiological importance ascribed to such organisms when isolated during disease. Because of the range of group agglutination, such organisms may even be agglutinated by the patients' serum as well.

**Frequency of Paratyphoid Infections.**—In this country the disease is relatively infrequent, though statistics are not available. It is frequent in Europe, especially in certain districts. In the present war the disease has been very common. In this country military camps have been favorable places for its development as seen in the outbreak of paratyphoid ("A") fever in the militia at the Mexican border.

**Communicability.**—The individual case and the carrier either through contact or by the contamination of water and milk, etc., are the usual sources of infection for paratyphoid fever. Food infection, however contaminated, plays the important role in the development of the more acute types of disease. Infection has followed the consumption of the following foods: milk and milk products; fish; potato salad; variously prepared string beans. In water it is more capable of a saprophytic existence than the typhoid bacillus, and has been isolated more often.

Meats or edible organs from cattle, swine, horse, sheep, geese, as well as various products containing these, are most frequently associated with acute food poisoning or infection. Although contamination of meat may occur in other ways the important source in epidemics is meat from infected animals. Such meat may by contact infect meat from healthy animals. Pickled, salted, and smoked meat may be a source of infection. Cooking cannot be relied upon to kill the bacilli because of the slow penetration of the heat. It is occasionally found in the feces, meat, and organs of some of the domestic animals, although apparently healthy. It has also been isolated from meat products and market milk.

**B. Enteritidis.**—In human pathology this bacillus plays the same role as the *B. paratyphosus B.* Meat is the usual source of infection, although other foodstuffs have been the cause. The disease is almost always of the acute type (see above). It is found in the feces, urine, and blood. Chronic carriers have not been found, although the bacilli have been found in gall-stones, and probably do occur. They may be present in the stools of healthy persons exposed to infection. Contact infection occurs, but infrequently. The pathological lesions are the same as those caused by the *B. paratyphosus B.* Infection of domestic animals occurs and the dissemination of the bacillus is the same as for paratyphoid *B.* It has not been found in water, probably not in milk, and infrequently in meat products. In cultures it is similar to *B. paratyphosus B.* For methods of diagnosis see below.

**Virulence.**—Although varying in degree, the various members of the group are highly virulent for white mice, guinea-pigs, rabbits, and rats. Death usually results from a septicemia. Toxic substances are freely produced in fluid media, which are heat resistant. The *paratyphoid A.* is probably the least virulent of the group.

**Immunity.**—In experimental animals immunity is produced by feeding and by injection. Immunity is produced not only against the homologous strain but, as a rule, against related strains. It has been suggested that because of the cross-protection of the members of these groups and the production of group antibodies active against typhoid or paratyphoid when one or the other is injected that a mixed vaccine of the typhoid-paratyphoid group be used. (Vaccines, see Part III.)

**Differential Diagnosis of Members of the Groups.**—The cultural differentiation only separates the A type from the B and enteritidis types. The members of the latter two groups, whether from man or animals show differences, but definite cultural groups have not been established. Weiss and Rice found that inositol fermentation was essentially a characteristic of *B. paratyphosus B.* Krumwiede, Pratt and Kohn, studying the reduction of fuchsin, the fermentation of xylose, arabinose and dulcitol, and also inositol, found that a grouping results which is highly suggestive in relation to some types and especially to host origin. The differences found, however, did not in many instances correlate with the agglutinative relationships. Much more work is necessary before the significance of such differences can be decided.

Immunity reactions, such as agglutination, fail to separate the members within the different groups, although the different groups are easily separated. As with other allied bacteria, however, group agglutinins are present. Other immune reactions give the same results.

**Variation in Types.**—The related enteritidis-paratyphoid strains show a wide ability for variation, not only culturally but in their pathogenicity and virulence. The above-described differences hold good for strains isolated from natural infections. There is reason to believe that a strain from man can adapt itself and develop a full degree of virulence for an animal host, and *vice versa*.

Even its original agglutination reactions may vary in degree, so that there is an apparent shifting from the paratyphoid to the enteritidis type; the antigenic properties do not change, however, in a parallel manner. In the study of the group, therefore, this should not be lost sight of and differential value thereby given to what may be variant characters. Even such characters as gas production may be lost or be extremely variable. Much work is needed before we shall be able to differentiate between dominant constant characters on which a classification can be based and variable characters, and also learn the range of variability. The extraordinary degree of cross-agglutination which may occur even with totally dissimilar types has been shown by Smith and Ten Broek. Because of adaptability of the members of the group the presence of any organism belonging to this group should be looked upon as a potential source of infection for man.

**Diagnostic Methods.**—The diagnostic methods are the Widal reaction, blood culture, isolation from the stool or urine or other sites of infection. The use of the Widal reaction for a differential diagnosis, from typhoid or between infection with different members of the group, may occasionally lead to errors because of the presence of group agglutinins. The titre of the serum should be determined against each of the types, that type which is agglutinated in the highest dilution, being the probable infecting organism. For surety of diagnosis the other methods should be employed. Their application is the same as in typhoid fever. For final diagnosis of the cultures isolated, immune sera against each of the types is necessary. The range of group agglutination of the sera used must be known before they are used for identification.

**Paratyphoid-like Bacilli.**—**B. Paratyphosus C.**—Bacilli having all characteristics of the paratyphoid group, but not agglutinated by paratyphoid or enteritidis serum, have been isolated from swine suffering from hog cholera, from man, meat, etc. The paratyphoid-like organisms found in feces, etc., but without pathological significance are frequently spoken of as belonging to this group. It seems to us better to reserve the term for the known pathogenic types.

**Types of the Paratyphoid-enteritidis Group Found in Animal Diseases.**—**B. Paratyphosus B. Types.**—*B. suispestifer*, hog-cholera bacillus commonly present as a secondary invader. The disease is caused by a filtrable virus. It is commonly encountered in food poisoning in man.

*B. typhi murium*, mouse typhoid bacillus, is used for the destruction of mice, successfully only when the technical details for the maintenance of virulence are carefully carried out. Instances of infection in man have been reported, and in Prussia its use is restricted by law.

*B. psittacosis*, the cause of enteritis in parrots, infectious for man, the disease being spoken of as psittacosis.

*Pseudotuberculosis of Guinea-pigs.*—A paratyphoid bacillus is found in the pseudotubercular lesions in the spleen and liver and lymph nodes. Sporadic cases or epidemics are common. Recently the condition developed in a series of guinea-pigs which we had injected with milk to test for the presence of tubercle bacilli. Unless careful examination is made confusion with tuberculous lesions might occur.

**Enteritidis Types.**—**Rat Viruses.**—Danyz isolated an organism from an epidemic of mice which had a high degree of virulence for rats and mice. It has been widely used for the extermination of rodents. Similar strains have been isolated by others. They are obtainable in the market as Ratin, Danyz-Virus, Liverpool Virus, etc. Recently infection in man has been reported. Domestic animals, meat and meat products should be protected from contact with the virus.

**Other Types.**—*Infectious Abortion in Mares.*—Various investigators have isolated paratyphoid types from infected mares which apparently constitute a distinct group.

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## CHAPTER XXV.

### DYSENTERY GROUP.

DYSENTERY may be divided into acute and chronic. Amebæ appear to be the chief exciting factor in most cases of chronic dysentery, though bacilli of the colon group also play a part.

In temperate climates acute dysentery is but very rarely due to amebæ, but usually to the bacilli identified by Shiga or to allied strains identified by Kruse, Flexner, Park, Hiss, Strong and others. The usual summer diarrheas are not excited by the dysentery bacilli.

**Historical Note.**—In 1897 Shiga found in the stools of cases of dysentery a bacillus which had not been before identified. In 1900 Flexner and Strong isolated bacilli which they at that time considered the same as those isolated by Shiga. In the same year Kruse, in Germany, isolated bacilli from cases of asylum dysentery which differed, however, in their agglutinative properties. Park and Dunham (1902) isolated a bacillus from a severe case of dysentery during an epidemic at Seal Harbor, Mt. Desert, Maine, which they showed differed from the Shiga dysentery bacillus in that it produced indol and differed in agglutinating characteristics. At first they considered it the same as the Flexner strain, but it was shown later by Park to be a distinct variety, and later found by him in widely separated epidemics.

Martini and Lentz, in December, 1902, found that the Shiga type was present in separate epidemics in Europe, but also in some cases, that other types similar to those isolated by Flexner, Park, Kruse, and others were found. These types differed from the Shiga type in fermenting mannite and in agglutination. In January, 1903, Hiss and Russel showed that a strain isolated by them differed from the Shiga type in the same characteristics.

German observers at first were inclined to consider the Shiga type as the only one producing dysentery, while the American observers considered both types of equal importance. Park investigated several epidemics and isolated only the Shiga type from some, from others either the Park-Hiss or the Flexner types, thus definitely proving the importance of the mannite fermenting types. The results obtained by others later were the same, so that no doubt exists that all of the types produce true dysentery.

**Morphological and Cultural Characteristics of Dysentery Bacilli.**—**Microscopic.**—Similar to bacilli of the colon group.

**Staining.**—Similar to bacilli of the colon group.

**Motility.**—No definite motility has been observed. The molecular movement is very active. Flagella are absent.

**Appearance of Cultures.**—On *gelatin* the colonies appear more like the typhoid than the colon bacilli. Gelatin is not liquefied. On agar growth is somewhat more delicate than that of the average colon cultures.

*On Potato.*—A delicate growth just visible or distinctly brownish.

*In Bouillon.*—Diffuse cloudiness with slight deposit and sometimes a pellicle.

See p. 324 for comparison with other members of colon-typhoid group.

The fermentation reactions vary, and on this basis the dysentery group is divided into subgroups and types. The first three groups are encountered in the United States. The last type is of infrequent occurrence but is accepted as a separate type by Shiga and by Lentz.

	Glucose.	Mannite.	Maltose.	Saccharose.	Indol.
<i>B. dysenteriae</i> . . . . .	+	—	—	—	—
<i>B. paradysenteriae</i> :					
Type 1 (Park-Hiss) . . . .	+	+	—	—	+
Type 2 (Flexner) . . . . .	+	+	+	—	+
Type 3 (Strong) . . . . .	+	+	—	+	+

In differential tests 2 per cent. mannite and 2.5 per cent. maltose give surer and more prompt reactions than the usual 1 per cent. The fermentations as given are only of differential value with freshly isolated cultures. After artificial cultivation as noted by Hiss and by Lentz the Park-Hiss strains may ferment maltose, and the Flexner strains, saccharose.



FIG. 134.—Dysentery bacilli.  $\times 1000$  diameters.



FIG. 135.—Colony of dysentery bacilli in gelatin.  $\times 40$  diameters.

**Pathogenesis. — Animal Tests.**—No characteristic lesions with one exception have followed the feeding of large quantities of bacilli. Dogs at times have diarrhea with slimy stools, but autopsy shows merely a hyperemia of the small intestine. The disease can be produced and occurs spontaneously in monkeys. Many animals are very sensitive to dead or living bacilli injected into the subcutaneous tissues, vein or peritoneal cavity.

The autopsy of animals dying quickly from injection into the peritoneum of living or dead bacilli shows the peritoneum to be hyperemic, the cavity more or less filled with serous or bloody serous exudate. The spleen is sometimes moderately swollen. The small intestine is filled with fluid, the large intestine is usually empty. The mucous membrane of both is hyperemic and sometimes contains hemorrhages. Conradi found ulcer formation in one case.

Subcutaneous injections of dead or living cultures are followed by infiltration of tissues and frequently by abscess formation. The dysentery bacilli are not found in the blood or organs of animals.

**Toxins.**—A highly poisonous endotoxin is found in autolysates and in filtered broth cultures. When injected into animals death results and on autopsy the same lesions are found as following the injection of live or dead bacilli. This endotoxin can be neutralized by large doses of immune serum. Kraus and others claim to have demonstrated the presence of an extracellular toxin against which an antitoxin can be produced. The Shiga type is the more toxic, the others less so or irregularly so.

**In Man.**—The etiological significance of the dysentery bacillus in man is not only shown by its constant presence and by the presence of immune bodies in the serum of infected persons, but also by experimental infection of man. Thus Strong infected two condemned criminals with pure cultures. Jehle infected himself. Kruse reported two accidental laboratory infections, and others have occurred.

As a general rule infection with the Shiga type is more severe and the mortality higher than infection from other types. But the severity of individual cases varies widely during an epidemic.

**Prevalence of the Disease.**—The disease is distributed over the whole world. The Shiga type and Type I of the paradysentery varieties are most commonly found in the United States.

**Character of Disease in Man.**—In the onset, acute dysentery is sudden and ushered in by cramps, diarrhea, and tenesmus. Bacillary dysentery is a disease especially of the mucous membrane of the large intestines. The epithelium is chiefly involved. In the lightest cases a catarrhal inflammation alone is present, in the more severe the lymph follicles are swollen and some necrosis of epithelium takes place.

In severe cases in adults the lesions are of a diphtheritic character and may be very marked. The entire lumen of the intestines may be filled with a fibrinous mass of pseudomembrane. In young children, even in fatal cases, the lesions may be more superficial.

**Distribution of the Bacilli.**—The bacilli are only found in the intestines. They do not invade the rest of the body. The feces, therefore, are the only excretions containing them.

**Duration of Life Outside of the Body.**—They usually die in stools in one to two days. In water they die out in several days to a week, exceptionally after a longer time.

**Communicability.**—The infected person is mainly responsible for the spread of dysentery. Especially dangerous are the mild or subacute cases

or carriers. Such infection may be direct or it may be indirect through contamination of food, dishes, linen, and clothes; water may also be infected and be the cause of epidemics. The bacillus has been isolated from water in such epidemics.

**Bacillus Carriers.**—Both healthy and convalescent carriers are found. The former may be equal to one-fourth or even one-half of the number of cases. Convalescents commonly excrete the bacilli for weeks and some become chronic carriers. The excretion is irregular, and slight relapses occur, when the bacilli are more numerous. The importance of finding and isolating the carriers during an epidemic is obvious. This is not always possible and general precautions against infection should be taken.

**Susceptibility.**—The frequent occurrence of healthy carriers and mild cases along with severe and fatal cases shows the varying resistance to infection. Disturbances of digestion and other conditions lowering the general resistance, such as heat and fatigue, are factors in infection.

**Immunity.**—Immune bodies appear in the blood some time after infection. In animals, immune bodies are also produced by injection. Agglutinins, bactericidal substances, precipitins, and opsonins are found. Neutralization of the toxic products is also possible with immune sera. True antitoxin is also probably produced.

Active immunity in animals is produced with difficulty against the Shiga type because of its high toxicity. Against the other types, however, immunity is more easily produced.

**Vaccines and Serums.**—See Part III.

**Diagnosis.**—The use of the Widal reaction is limited, because the symptoms are sufficiently diagnostic in well-marked cases and, further, the reaction does not appear during the early acute stage. Later it may be used. Group or normal agglutinins are commonly present and interfere greatly with the value of the reaction in the paradysentery types. The action on the infecting subgroup is usually sharp, but infection by the types of the paradysentery group can frequently not be differentiated. An agglutination reaction is commonly present in chronic carriers. For even moderate diagnostic value the serum should agglutinate in 1 to 50 in infections with *B. dysenteriae* and in 1 to 100 in other types.

Isolation of the bacillus is the only method of diagnosis for identification of the type causing the infection. The mucous flakes in the stool should be selected for plating. The methods of isolation and identification are the same as for typhoid or paratyphoid, except that the crystal violet should be omitted from the Conradi medium as the growth of many strains is inhibited more or less by aniline dyes. The bacilli are so abundant in the mucus of most cases that they can be readily isolated from nutrient agar plates. If Endo is used the plates should be fresh. When the color returns the growth of the Shiga types is usually inhibited.

**Differential Diagnosis of Type.**—The cultural differences have been given. The actual nature of the bacillus should be verified by agglu-



tion, although the cultural characters will suffice for a tentative diagnosis from a case clinically typical.

**Agglutination.**—Sera from rabbits or goats are used, as horses develop normal and group agglutinins to a high degree.

For practical purposes two sera, one for *B. dysenteriae* and a polyvalent serum for the paradysentery types may be used, further differentiation being determined culturally. In the use of sera for differentiation of the latter groups univalent sera for each of the types must be on hand and all the precautions against error from group agglutinins should be observed. Variations in agglutinability of freshly isolated strains adds to the difficulty.

**Variability.**—Cultures, after prolonged artificial cultivation, may show variations in their sugar fermentations. By passage through animals (monkey, etc.), the original characteristics are usually restored.

## CHAPTER XXVI.

### BACILLUS PYOCYANEUS (BACILLUS OF GREEN AND OF BLUE PUS). BACILLUS PROTEUS (VULGARIS).

#### BACILLUS PYOCYANEUS.

THE blue and green coloration which is occasionally found to accompany the purulent discharges from open wounds is usually due to the action of the *Bacillus pyocyaneus*. It was first obtained in pure culture and its significance noted by Gessard, 1882.

**Morphology.**—Slender rods from  $0.3\mu$  to  $1\mu$  broad and from  $2\mu$  to  $6\mu$  long; frequently united in pairs or in chains of four to six elements; occasionally growing out into long filaments and twisted spirals. The bacillus is actively motile, a single flagellum being attached to one end. Does not form spores. *Stains* with the ordinary aniline colors; does not stain with Gram's stain.

**Biology.**—Aërobic, facultative anaërobic, liquefying, motile bacillus. Growing anaërobically it produces no pigment. Grows readily on all artificial culture media at room temperature, though best at  $37^{\circ}$  C., and gives to some of them a bright green color in the presence of oxygen. In *gelatin-plate* cultures the colonies are rapidly developed, imparting to the medium a fluorescent green color; liquefaction begins at the end of two or three days, and by the fifth day the gelatin is usually entirely liquefied. The deep colonies, before liquefaction sets in, appear as round, granular masses with scalloped margins, having a yellowish-green color; the surface colonies have a darker green centre, surrounded by a delicate, radiating zone. In *stick cultures in gelatin*, liquefaction occurs at first near the surface, in the form of a small funnel and gradually extends downward; later the liquefied gelatin is separated from the solid part of the medium by a horizontal plane, a greenish-yellow color being imparted to that portion which is in contact with the air. On *agar* a wrinkled, moist, greenish-white layer is developed, while the surrounding medium is bright green; this subsequently becomes darker in color, changing to blue green or almost black. In *bouillon* the green color is produced, and the growth appears as a delicate, flocculent sediment. *Milk* is coagulated and assumes a yellowish-green color.



FIG. 136.—*Bacillus pyocyaneus*. (From Kolle and Wassermann.)

**Pigment.**—Two pigments are produced by this bacillus—one of a fluorescent green which is common to many bacteria. This is soluble in water but not in chloroform. The other (pyocyanin) of a blue color is soluble in chloroform, and may be obtained from pure solution in long, blue needles. This pigment distinguishes the *Bacillus pyocyaneus* from other fluorescing bacteria. Pigment production is usually more marked with incubation at 22° C. The ability to produce pigment may be lessened or lost by artificial cultivation.

**Ferment.**—Besides the ferment causing liquefaction of gelatin there is one which acts on albumin. It resists heat. This ferment called pyocyanase is able to dissolve bacteria, and it has been stated to have some protective power when injected into animals. It has been used locally in diphtheria in a number of cases. We think it has no advantage over the cleansing preparations.

**Distribution.**—This bacillus is very widely distributed in nature; it is frequently found on the healthy skin of man, in the feces of many animals, in water contaminated by animal or human material, in purulent discharges, and in serous wound secretions.

**Pathogenesis.**—Its pathogenic effects on animals have been carefully studied. It is pathogenic for guinea-pigs and rabbits. Subcutaneous or intraperitoneal injections of 1 c.c. or more of a bouillon culture usually cause the death of the animal in from twenty-four to thirty-six hours. Subcutaneous inoculations produce an extensive inflammatory edema and purulent infiltration of the tissues; a sero-fibrinous or purulent peritonitis is induced by the introduction of the bacillus into the peritoneal cavity. The bacilli multiply in the body and may be found in the serous or purulent fluid in the subcutaneous tissues or abdominal cavity, as well as in the blood and various organs. When smaller quantities are injected subcutaneously the animal usually recovers, only a local inflammatory reaction being set up (abscess), and the animal is subsequently immune against a second inoculation with doses which would prove fatal to an unprotected animal. Loew and Emmerich have shown that the enzymes produced in the pyocyaneus cultures are capable of destroying many forms of bacteria in the test-tube, and have a slight protecting value in the body.

Its presence in wounds in man greatly delays the process of repair, and may give rise to a general depression of the vital powers from the absorption of its toxic products. This bacillus has been obtained in pure culture from pus derived from the tympanic cavity in disease of the middle ear, from cases of ophthalmia, and bronchopneumonia. Kruse and Pasquale have found the organism in three cases of idiopathic abscess of the liver, in two of them in immense numbers and in pure culture. Ernst and Schürmayer report the presence of the bacillus pyocyaneus in serous inflammations of the pericardial sac and of the knee-joint. Ehlers gives the history of a disease in two sisters who were attacked simultaneously with fever, albuminuria, and paralysis. It was thought that they would prove to have typhoid fever or meningitis, but on the twelfth day there was an eruption of blisters, from the contents of which the *Bacillus pyocyaneus* was isolated. Krambals refers to

seven cases in which a general pyocyanus infection occurred, and adds an eighth from his own experience. In this the *Bacillus pyocyanus* was obtained postmortem from green pus in the pleural cavity, from serum in the pericardial sac, and from the spleen in pure culture. Schimmelbusch states that a physician injected 0.5 c.c. of sterilized (by heat) culture into his forearm. As a result of this injection, after a few hours he had a slight chill, followed by fever, which at the end of twelve hours reached 38.8° C.; an erysipelatous-like swelling of the forearm occurred, and the glands in the axilla were swollen and painful. Wassermann reports an epidemic of septic infection of the newborn, starting in the umbilicus. In all there were eleven deaths. Lartigau found it in well water, and in great abundance in the intestinal discharges of a number of cases made ill by drinking the water. It has also been found in a certain number of cases of gastro-enteritis in which no special cause of infection could be noted.

We may therefore conclude from these facts that the *Bacillus pyocyanus*, although ordinarily but slightly pathogenic for man, may under certain conditions, as in general debility, become a dangerous source of infection. Children would seem to be particularly susceptible.

**Differential Diagnosis of the Pyocyanus from other Fluorescing Bacteria.**—This is easy enough as long as it retains its pigment-producing property. When an agar culture is agitated with chloroform a blue coloration demonstrates the presence of this bacillus. When the pyocyanin is no longer formed, however, the diagnosis is by no means easy, particularly when the pathogenic properties are also gone.

**Immunity.**—Animal infection is followed by the production of anti-toxic and bactericidal substances.

### BACILLUS PROTEUS (VULGARIS).

The term *B. proteus* is used for a group of bacilli classed as putrefactive bacteria, because they decompose protein substances with the production of a disagreeable odor. They were discovered by Hauser in 1885. The limits of this group are not well defined nor has it been determined how many varieties there are in this group. The following is a description of a typical liquefying variety.

**Morphology.**—Bacilli varying greatly in size; most commonly occurring 0.6 $\mu$  broad and 1.2 $\mu$  long, but shorter and longer forms may also be seen, even growing out into flexible filaments which are sometimes more or less wavy or twisted like braids of hair.

The bacillus does not form spores, and *stains* readily with fuchsin or gentian violet. It is Gram-negative.

**Biology.**—An aërobic, facultative anaërobic, liquefying, motile bacillus. Grows rapidly in the usual culture media at room temperature.

**Growth on Gelatin.**—The growth upon *gelatin plates* containing 5 per cent. of gelatin is very characteristic. At the end of ten to twelve hours at room temperature small round depressions in the gelatin are observed which contain liquefied gelatin and a whitish mass consisting of bacilli in the centre. Under a low-power lens these depres-

sions are seen to be surrounded by a radiating zone composed of two or more layers, outside of which is a zone of a single layer, from which ameba-like processes extend upon the surface of the gelatin. These processes are constantly undergoing changes in their form and position. The young colonies deep down in the gelatin are somewhat more compact, and rounded or hump-backed; later they are covered with soft down; then they form irregular, radiating masses, and simulate the superficial colonies. When the consistency of the medium is more solid, as in 10 per cent. gelatin the liquefaction and migration of surface colonies are more or less retarded. In *gelatin-stick* cultures liquefaction take place rapidly along the line of puncture, and soon the entire contents of the tube are liquefied.

Upon *nutrient agar* a rapidly spreading, moist, thin, grayish-white layer appears, and migration of the colonies also occurs. *Milk* is coagulated, with the production of acid.

Cultures in media containing albumin or gelatin have a disagreeable, putrefactive odor, and become alkaline in reaction. Growth is most luxuriant at a temperature of 24° C., but is plentiful also at 37° C. It is an *aërobic* bacillus but it grows also in the absence of oxygen. In the latter condition it loses its power of liquefying gelatin. It produces indol and phenol from peptone solutions. The proteus develops fairly well in urine, and decomposes urea into carbonate of ammonia.

**Pathogenesis.**—This bacillus is pathogenic for rabbits and guinea-pigs when injected in large quantities into the circulation, the abdominal cavity, or subcutaneously, producing death with symptoms of poisoning. Hauser has obtained the *Bacillus proteus (vulgaris)* from a case of purulent peritonitis, from purulent puerperal endometritis, and from a phlegmonous inflammation of the hand.

It is probable that in some instances food poisoning has been due to the contamination of foods by *B. proteus*. Because of the proteolytic power, toxic products "ptomaines" may develop as a result of its growth. Under these conditions decomposition has started and the food is disagreeable both in taste and odor and for this reason food poisoning of this type is probably much more uncommon than that due to members of the paratyphoid-enteritidis group, or to *B. botulinus* where there is no change or only a slight change in odor and taste.

*Proteus vulgaris* has been found to be the predominating organism in the alvine discharge in cases of cholera infantum. The prominent symptoms in these cases were drowsiness, stupor, and great reduction in flesh, more or less collapse, frequent vomiting and purging, with watery and generally offensive stools.

The *Proteus vulgaris* appears to be next in importance to the *Bacillus communis* in the etiology of cystitis and pyelonephritis.

The *Proteus vulgaris* is usually a harmless parasite when located in the mucous membrane of the nasal cavities. Here it only decomposes the secretions, with the production of a putrefactive odor. It is found occasionally in the discharge from cases of otitis media in association with other bacteria.

## CHAPTER XXVII.

### THE BACILLUS AND THE BACTERIOLOGY OF TUBERCULOSIS.

**Historical Note.**—A knowledge of phthisis was certainly present among men at the time from which our earliest medical descriptions come. For over two thousand years many of the clearest thinking physicians have considered it a communicable disease; but it is only within comparatively recent times that the infectiousness of tuberculosis has become an established fact in scientific medicine. Villemin, in 1865, by infecting a series of animals through inoculations with tuberculous tissue, showed that tuberculosis might be induced, and that such tissue carried the exciting agent of the disease. He also noticed the difference in virulence between tuberculous material of human and bovine sources, and says that not one of the rabbits inoculated with human material showed such a rapidly progressive and widespread generalization as those receiving material from the cow. Baumgarten demonstrated, early in 1882, bacilli in tissue sections which are now known to have been tubercle bacilli. But these investigations and those of others at the same time, though paving the way to a better knowledge of the disease, proved to be unsatisfactory and incomplete. The announcement of the discovery of the tubercle bacillus was made by Koch in March, 1882. Along with the announcement satisfactory experimental evidence was presented as to its etiological relation to tuberculosis in man and in susceptible animals, and its principal biological characters were given. He submitted his full report in 1884. Innumerable investigators now followed Koch into this field, but their observations served only to confirm his discovery.

**Distribution of Bacilli.**—They are found in the sputum of persons and animals suffering from pulmonary or laryngeal tuberculosis, either free or in the interior of pus cells; in miliary tubercles and fresh caseous masses in the lungs and elsewhere; in recent tuberculous cavities in the lungs; in tuberculous glands, joints, bones, serous effusions, mucous membranes, and skin affections. They are also found in the feces of those suffering from tuberculosis of the intestines or of those who have swallowed tuberculous sputum. They are frequently present in the blood in very small numbers, in large number only in acute miliary tuberculosis.

**Morphology.**—The tubercle bacilli are slender, non-motile rods of about  $0.3\mu$  in diameter by  $1.5\mu$  to  $4\mu$  in length. (Plate VI, Figs. 1 and 2.) The morphology is extremely variable, especially on culture media, and varies with the type of medium used. Commonly they occur singly or in pairs, and are then usually slightly curved; frequently they are observed in smaller or larger bunches. Under exceptional conditions branching and club-shaped forms are observed. The tubercle bacillus is probably closely allied to nocardia. In stained preparations there are often seen unstained portions. In old cultures irregular forms may develop, the rods being occasionally swollen at one end or presenting

lateral projections. Here also spherical granules appear which stain with more difficulty than the rest of the bacillus and also retain the stain with greater tenacity. The bacilli, however, containing these bodies are not appreciably more resistant than those not having them; therefore they cannot be considered true spores. The bacilli have a thin capsule, shown in one way by the fact that they appear thicker when stained with fuchsin than with methylene blue.

**Chemical Constituents of the Tubercle Bacilli.**—Water 86 per cent.; dry substance 14 per cent., 25 per cent. of which is soluble in alcohol and ether, consisting of free fatty acids and fatty acids combined with the higher alcohol "mykol" to form a wax; lecithin proteins, other nucleo-albumins, and inorganic bases constitute the remainder.

**Staining Peculiarities.**—These are very important, for by them its recognition in microscopic preparations of sputum, etc., is rendered possible. Owing to content of waxy substance it does not readily take up the ordinary aniline colors, but when once stained it is very difficult to decolorize, even by the use of strong acids. The more recently formed bacilli are much more easily stained and decolorized than the older forms. For methods of staining see pp. 77 and 80.

**Biology.**—The bacillus of tuberculosis is a *parasitic, aerobic, non-motile* bacillus, and grows best at a temperature of about 37° C., limits 30° to 42° C. It does not form true spores.

**Resistance.**—The bacilli, because of the protection given by their waxy substances, it has been assumed, have a somewhat greater resisting power than most other pathogenic bacteria. Frequently a few out of a great number of bacilli resist desiccation at ordinary temperatures for months; most bacilli die, however, soon after drying. There is a greater resistance shown by the tubercle bacillus than by most other non-spore-bearing bacilli to the action of the products of bacterial growth as in souring milk, in water, and in sewage. In water and sewage they may remain viable for weeks. They frequently retain their vitality for several weeks, or even months, in putrefying material, such as sputum. In cultures the bacilli do not live longer than three months,

#### EXPLANATION OF PLATE VI.

FIG. 1.—Tuberculous lymph node "giant cell" containing tubercle bacilli "human type." Bacilli red, rest of specimen blue. Ziehl-Neelsen stain.  $\times 1000$  diam.

FIG. 2.—Tuberculous sputum from human case. Stain same as above.  $\times 1000$  diam.

FIG. 3.—Tuberculous sputum, human case. Stained by Hermann's method. Tubercle bacilli violet, rest of specimen brown.

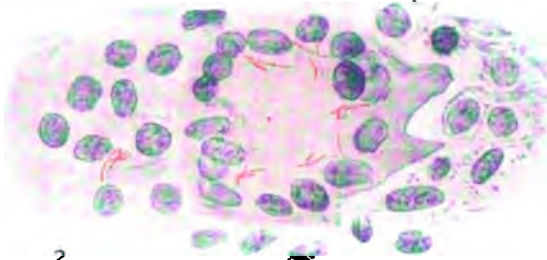
FIG. 4.—Pus from tuberculous abscess in cow. "Bovine type" of bacillus. Stained same as Figs. 1 and 2.  $\times 1000$  diam.

FIG. 5.—Section through leprous skin showing bacilli in clumps in and out of cells and large "leprous cell" containing a ball of bacilli. Stained with Ziehl-Neelsen.

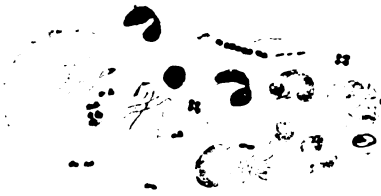
FIG. 6.—Photograph of human type of tubercle bacilli from sputum. Bacilli in red, rest of specimen blue.  $\times 1000$  diam. (Fränkel and Pfeiffer.)

PLATE VI

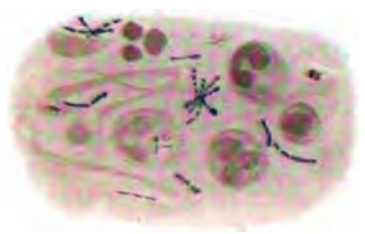
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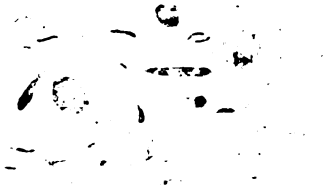
2



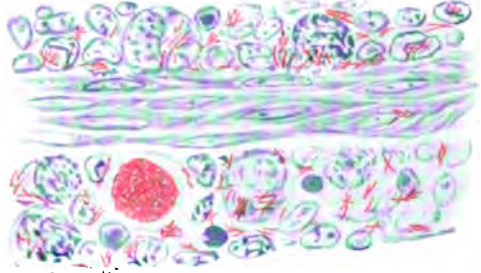
3



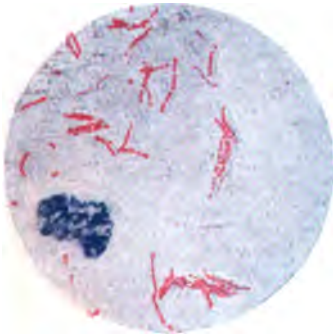
4



5



6







unless the media be favorable, such as egg or serum; transplants after this time may fail to grow. A few bacilli, sufficient to infect guinea-pigs, may persist much longer. Cold has little effect upon them. When dry, some of the organisms stand dry heat at  $100^{\circ}$  C. for twenty minutes but are dead in forty-five minutes; but when in fluids and separated as in milk, they are quickly killed—viz., at  $60^{\circ}$  C. in twenty minutes, at  $65^{\circ}$  C. in fifteen minutes, at  $70^{\circ}$  C. the great majority in one minute, all in five minutes, at  $80^{\circ}$  C. the great majority in one-half minute, all in one minute, and at  $95^{\circ}$  C. in one-half minute. In some experiments they appear to withstand a higher temperature. As pointed out by Theobald Smith, when milk is heated in a test-tube in the usual way the cream which rises on heating is exposed on its surface to a lower temperature than the rest of the milk, and as this contains a large percentage of the bacteria some of them are exposed to less heat than those in the rest of the fluid. Rosenau points out another source of error: If a moderate number of killed bacilli are injected, limited lesions will arise and caseation may follow. On killing and autopsying the animals, tubercle bacilli can then be demonstrated in smears from the lesions, and the inoculation is considered positive. If, however, this material is reinjected into a second pig, the latter will show nothing on autopsy. This capacity of dead bacilli to cause macroscopic lesions has long been shown by Prudden and Hodenpyl. Its importance, however, is not sufficiently considered.

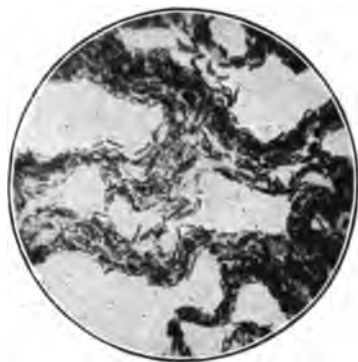


FIG. 137.—Tubercle bacilli. Impression preparation from small colony on coagulated blood serum.  $\times 1000$  diameters.

The resisting power of this bacillus to chemical disinfectants, drying, and light is considerable, but not as great as it is apt to appear, for, as in sputum, the bacillus is usually protected by mucus or cell protoplasm from penetration by the germicidal agent. It is not always destroyed by the gastric juice in the stomach, as is shown by successful infection experiments in susceptible animals by feeding them with tubercle bacilli. They are destroyed in sputum in six hours or less by the addition of an equal quantity of a 5 per cent. solution of carbolic acid. Bichloride of mercury is less suitable for the disinfection of sputum as it combines with the mucus and forms a more or less protecting envelope. Iodoform has no effect upon cultures until 5 per cent. is added. The fumes from four pounds of burning sulphur to each 1000 cubic feet of air space will kill tubercle bacilli in eight hours when fully exposed to the action of the gas, providing they are moist, or abundant moisture is present in the air. Formaldehyde gas is quicker in its action, but not much more efficient. Ten ounces of formalin should be employed for each 1000 cubic feet of air space. The tubercle bacillus resists the action of alkaline hypo-

chloride solution ("antiformin") in dilutions which quickly dissolve non-acid fast bacteria.

The tubercle bacillus in sputum when exposed to direct sunlight is killed in from a few minutes to several hours, according to the thickness of the layer and the season of the year; it is also usually destroyed by diffuse daylight in from five to ten days when placed near a window in fine powder. Protected in cloth the bacilli survive exposure to light for longer periods. Tuberculous sputum expectorated upon sidewalks, etc., when left undisturbed in the shade may be infectious for weeks, but when exposed to the action of direct sunlight will, in many cases, especially in summer, be disinfected by the time it is in condition to be carried into the air as dust, but not before children and flies have an opportunity of getting into it. The action of sunlight and other more important hygienic reasons suggest that the consumptive patients should occupy light, sunny rooms.

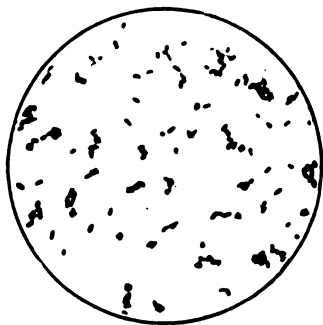


FIG. 138.—Tubercle bacilli, bovine.  
× 1000 diameters.

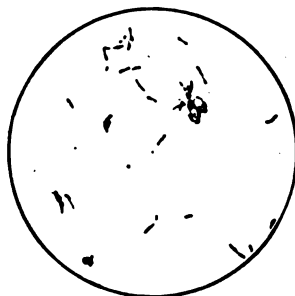


FIG. 139.—Tubercle bacilli, human.  
× 1000 diameters.

Dried sputum in rooms protected from abundant light has occasionally been found to contain virulent tubercle bacilli for as long as ten months. For a year at least it should be considered dangerous. The Röntgen rays have a deleterious effect on tubercle bacilli in cultures, but practically none upon those in tissues.

**Multiplication of Tubercle Bacilli in Nature Takes Place Only in the Living Animal.**—The tubercle bacillus is a strict parasite—that is to say, its biological characters are such that it could scarcely find natural conditions outside of the bodies of living animals favorable for its multiplication. Under exceptional conditions, such as in freshly expectorated sputum, tubercle bacilli may increase for a limited time.

**Cultivation of the Tubercle Bacillus.**—On account of their slow growth and the special conditions which they require, tubercle bacilli cannot be grown in pure culture by the usual plate method on ordinary culture media. Koch first succeeded in cultivating and isolating this bacillus on coagulated beef serum, which he inoculated by carefully rubbing the surface with sections of tuberculous tissue and then leaving

the culture, protected from evaporation, for several weeks in the incubator. Cultures are more readily obtained of human or avian than of bovine bacilli.

**Growth on Coagulated Dog or Bovine Serum or on Egg.**—On these, one of which is generally used to obtain the first culture, the growth is usually visible at the end of ten days at 37° C., and at the end of three or four weeks a distinct and characteristic development has occurred. On serum small, grayish-white points and scales first appear on the surface of the medium. As development progresses there is formed an irregular, membranous-looking layer. On egg the growth is in the form of more or less elevated colonies which may become confluent.

**Growth on Nutrient 3 to 5 Per Cent. Glycerin Agar.**—Owing to the greater facility of preparing and sterilizing *glycerin agar*, it is now usually employed in preference to blood serum for continuing to produce later cultures. When numerous bacilli have been distributed over the surface of the culture medium, a rather uniform, thick, white layer, which subsequently acquires a slight yellowish tint, is developed; when the bacilli sown are few in number, or are associated in scattered groups, separate colonies are developed, which acquire considerable thickness and have more or less irregular outlines. The growth appears similar to that shown upon bouillon as seen in Fig. 140.



FIG. 140.—Growth of tubercle bacilli upon glycerin bouillon. (Kolle and Wasserman.)

**Growth on Nutrient Veal or Beef Broth Containing 5 Per Cent. of Glycerin.**—Glycerin broth is used for the development of tuberculin and must be neutral to litmus, viz., between 1.5 and 2 per cent. acid to phenolphthalein. On these media the tubercle bacillus grows readily if a very fresh thin film of growth from the glycerin agar or a small piece of pellicle removed from a previous broth culture is floated on the surface. This continues to enlarge as long as it floats on the surface of the liquid, and in the course of three to six weeks covers it wholly as a single film, which on agitation is easily broken up and then settles to the bottom of the flask, where it ceases to develop further. The liquid remains clear. A practical point of importance, if a quick growth is desired, is to use for the new cultures a portion of the pellicle of a growing bouillon culture, which is very thin and actively increasing.

**Growth on Potato.**—A good growth from cultures and sometimes even from tissue takes place on potato, and this forms the most uniform medium for stock cultures.

**Obtaining of Pure Cultures of the Tubercle Bacillus from Sputum, Infected Tissue, and Other Materials.**—On account of the time required and the difficulties to be overcome, this is never desirable except when careful investigations of importance are to be undertaken. *Pure cultures* can be obtained directly from tuberculous material, if the tubercle bacilli are present in sufficient number and mixed infection is not present, by using the proper blood serum or egg culture medium (p. 106); but it is difficult to get material free from other bacteria which grow much more rapidly and take possession of the medium before the tubercle bacillus has had time to form visible colonies. It is usually necessary first to inoculate guinea-pigs, subcutaneously or intramuscularly, preferably in the thigh, and then obtain cultures from the animals as soon as the tuberculous infection has developed. In this way, due to the susceptibility of the guinea-pig to tuberculosis, cultures may be obtained from material containing very few tubercle bacilli, although contaminating bacteria may be very numerous.

Animals inoculated usually die at the end of three weeks to four months. It is better, however, not to wait until the death of the animals, but at the end of four to six weeks to kill a guinea-pig without violence, using illuminating gas, chloroform, or ether in a closed tin or jar. (Animals which develop tuberculosis acutely are apt to have abundant tubercle bacilli and give successful cultures, while the chronic cases usually have few bacilli and may give unsuccessful cultures.) The animal after being killed is tied out in trays, and after washing with a 5 per cent. solution of carbolic acid, immediately autopsied. The skin over the anterior portion of the body having been carefully turned back, the inguinal nodes are removed with fresh instruments. The nodes on the side of injection are especially favorable for cultures. The abdomen is then opened and the spleen and retroperitoneal nodes removed. As the organs are removed they should be placed in Petri dishes and thoroughly minced with knife and forceps. Fresh instruments should be used for each operation. The sternal nodes may be used for cultures, but the lungs are almost useless, as the majority of cultures will be contaminated. The minced tissue is then placed on the surface of the culture media, both egg and glycerin egg being used, and evenly and thoroughly smeared over its surface, then the cotton plug is dipped in hot paraffin to aid in keeping the media from drying. The tubes are incubated in an inclined position. On egg, growth is visible in from seven to ten days, and well marked at the end of three weeks. Many tubes should be inoculated, as it is only with the dexterity acquired by practice that contaminations are avoided. As will be noted further on, the growth of the bovine type will be very sparse and on glycerin egg probably negative.

Cultures may also be obtained with a fair proportion of successful results by the antiformin method or the method of Petroff. In the former (see p. 397) the washed sediment is inoculated on egg media. Petroff (see p. 106) digests sputum or feces with equal amounts of 3 per

cent. NaOH for one-half hour at 37° C., neutralizes with dilute HCl and inoculates the sediment obtained by centrifugalizing, on his special gentian-violet egg medium. A successful culture of an acid-fast bacillus does not necessarily mean a tubercle bacillus. Van Winkle in our laboratory has isolated two non-pathogenic acid-fast strains in this way from sputa.

**Pathogenesis.**—The tubercle bacillus is pathogenic not only for man, but for a large number of animals, such as the cow, monkey, pig, cat, etc. Young guinea-pigs are very susceptible, and are used for the detection of tubercle bacilli in suspected material. When inoculated with the minutest dose of the living bacilli they usually succumb to the disease. Infection is most rapidly produced by intraperitoneal injection. If a large dose is given, death follows in from ten to twenty days. The omentum is found to be clumped together in sausage-like masses which contain many bacilli. There is no serous fluid in the peritoneal cavity, but generally in both pleural sacs. The spleen is enlarged, and it, as well as the liver and peritoneum, contains large numbers of tubercle bacilli. If smaller doses are given, the disease is prolonged. The peritoneum and internal organs—spleen, liver, etc., and often the lungs—are then filled with tubercles. On subcutaneous injection, for instance, into the thigh, there is a thickening of the tissues about the point of inoculation, which may break down in one to three weeks and leave a sluggish ulcer covered with cheesy material. The neighboring lymph nodes are swollen, and at the end of two or three weeks may attain the size of hazel-nuts. Soon an irregular fever is set up, and the animal becomes emaciated, usually dying within four to eight weeks. If the injected material contained only a small number of bacilli the wound at the point of inoculation may heal up and death be postponed for a long time. At autopsy the lymphatic nodes are found to have undergone cheesy degeneration; the spleen may be very much enlarged, and throughout its substance, which is colored dark red, are distributed masses of nodules. The liver is also commonly increased in size, streaked brown and yellow, and the lungs are filled with grayish-white tubercles; but, as a rule, the kidneys contain no nodules. Occasionally the lesions are limited to the inguinal and retroperitoneal nodes. Tubercle bacilli are found in the affected tissues, but the more chronic the process the fewer the bacilli present.

Injection into the thigh is to be preferred for diagnostic purposes, the swelling of the local lymph nodes being then palpable. As soon as this is appreciable the node may be removed with or without killing the pig, the presence or absence of tuberculous lesions noted, and smears made for the detection of tubercle bacilli, thus saving time. It must be remembered that the pig may not show the usual picture of generalized tuberculosis, but only a swelling of the local lymph nodes. Fortunately tubercle bacilli are usually easily demonstrable in smears made from the crushed nodes. If there is any doubt the remaining tissue should be emulsified and reinjected into a second set of pigs. Another point to be considered is that other organisms may, rarely, give a picture difficult to distinguish macroscopically from tuberculosis,

as, for instance, streptothrix. To safeguard against error smears should be stained and tubercle bacilli demonstrated. Chronic guinea-pig septicemia may be accompanied by lesions in the spleen which might be taken for tuberculous lesions.

Rabbits are very susceptible to tuberculosis of the bovine type, less so to that of the human type. This will be given more in detail under the differences between human and bovine tuberculosis.

Monkeys are very susceptible to infection with both types of bacilli. Cats, dogs, rats, and mice are susceptible, the last two usually show no tuberculous lesions, but there is great multiplication of the bacilli in the tissues.

**Tubercle Toxins.**—The tubercle bacillus produces no true toxins. The bodies of the bacteria contain substances which cause necrosis of tissue with subsequent caseation or abscess. In broth cultures, after filtration, are present substances which produce fever and inflammatory reactions of tissues. These substances as well as extracts from tubercle bacilli are highly toxic for tuberculous animals, little if at all for normal animals, and cause fever only in the former and the tissue inflammation spoken of as marked about tuberculous lesions. These poisons will be considered in detail later in connection with tuberculins.

**Action upon the Tissues of the Poisons Produced by the Tubercle Bacillus.**—Soon after the introduction into the tissues of tubercle bacilli, either living or dead, the cells surrounding them begin to show that some irritant is acting upon them. The connective-tissue cells become swollen and undergo mitotic division, the resultant cells being distinguished by their large size and pale nuclei. A small focus of proliferated epithelioid cells is thus formed about the bacilli, and according to the intensity of the inflammation these cells are surrounded by a larger or smaller number of lymphoid cells. When living bacilli are present and multiplying the lesions progress, the central cells degenerate and die, and a cheesy mass results, which later may lead to the formation of cavities. Dead bacilli, on the other hand, unless bunched together give off sufficient poison to cause the less marked changes only (Prudden and Hodenpyl). Of the gross pathological lesions produced in man by the tubercle bacilli the most characteristic are small nodules, called miliary tubercles. When young, and before they have undergone degeneration, these tubercles are gray and translucent in color, somewhat smaller than a millet seed in size, and hard in consistence. But miliary tubercles are not the sole tuberculous products. The tubercle bacilli may cause diffuse growth of tissue identical in structure with that of miliary tubercles, that is, composed of a basement substance, containing epithelioid, giant, and lymphoid cells. This diffuse tuberculous tissue also tends to undergo cheesy degeneration.

**Point of Entrance of Infection.**—Infection by the tubercle bacillus takes place usually through the respiratory tract or the digestive tract, including the pharynx and tonsils, more rarely through wounds of the skin.

Tuberculosis may be considered to be caused chiefly by the direct transmission of tubercle bacilli to the mouth through soiled hands, lips, handkerchiefs, milk, etc., or by the inhalation of fine particles of mucus thrown off by coughing or loud speaking, or of dust contaminated by tuberculous sputum or feces.

**Tuberculosis of Skin and Mucous Membranes.**—When the skin or mucous membranes are superficially infected through wounds there may develop lupus, ulceration, or a nodular growth. The latter two forms of infection are apt after an interval to cause the involvement of the nearest lymphatic nodes.

**Tuberculosis of Respiratory Tract.**—The lungs are the most frequent location of clinically recognizable tuberculous inflammation. On account of their location they are greatly protected from external infection. Most of the bacilli are caught upon the nasal or pharyngeal mucous membranes. Only a small percentage can find their way to the larynx and trachea, and still less to the smaller bronchioles. From the examination of the lungs of miners as well as from experimental tests there is no doubt but that some of the bacilli may find their way into the deeper bronchi. The deeper the bacilli penetrate the more unlikely that they can be cast out. On the other hand, the lungs are the most likely point of localization of tubercle bacilli which find their way into the blood stream. It is in this way that the lungs frequently become infected. It is now well established that infection taking place through the intestine may find its way by the blood to the lungs and excite there the most extensive lesions with or without leaving any trace of its point of entrance. Even if infection of the lung is slight or entirely absent the tubercle bacilli then find their way to the bronchial nodes where lesions may develop. Lesions either active or latent, anywhere in the body may be the source of a subsequent pulmonary infection. Should bacilli find their way into the blood, infection of the lung tissue will result if the resistance is lowered for any reason. The nasal cavities are rarely affected with tuberculosis, but more often the retropharyngeal tissue. Tuberculosis of this tissue as well as that of the tonsils is apt to give rise to infection of the lymph nodes of the neck. It is believed that just as bacilli may pass through the intestinal walls to infect the mesenteric nodes, so bacilli may, without leaving any trace, pass through the tonsils to the nodes of the neck.

Primary infection of the larynx is rare. Secondary infection is fairly common. The region of the vocal cords and the interarytenoid space are the special sites attacked.

**Infection by Inhalation of Dried and Moist Bacilli.**—A common mode of infection is by means of tuberculous sputum, which, being coughed up by consumptives, is either disseminated as a fine spray and so inhaled, or, carelessly expectorated, dried and, broken up by tramping over it, sweeping, etc., distributes numerous virulent bacilli in the dust. As long as the sputum remains moist there is no danger of dust infection, but only of direct contact; it is when it becomes dry, as on handkerchiefs, bedclothes, and the floor, etc., that the dust is a source of danger.



A great number of the expectorated and dried tubercle bacilli undoubtedly die, especially, as we have said, when acted upon by direct sunlight; but when it is considered that as many as five billion virulent tubercle bacilli may be expectorated by a single tuberculous individual in twenty-four hours, it is evident that even a much smaller proportion than are known to stay alive will suffice in the immediate vicinity of consumptives to produce infection unless precautions are taken to prevent it. The danger of infection is greatest, of course, in the close neighborhood of tuberculous patients who expectorate profusely and indiscriminately, that is, without taking the necessary means for preventing infection. We found that of 100 tuberculous men admitted to one of the consumption hospitals, only 20 claimed to have taken any care to prevent the contamination of their surroundings by their sputum. There is much less danger of infection at a distance, as in the streets for instance, where the tubercle bacilli have become so diluted that they are less to be feared. In rooms the sputum is not only protected from the direct sunlight, but it is constantly broken up and blown about by the walking, closing of doors, etc. In crowded streets on windy days infected dust must sometimes be in the air unless the expectoration of consumptives is controlled.

Exhaustive experiments made by many observers have shown that particles of dust collected from the immediate neighborhood of consumptives, when inoculated into guinea-pigs, produce tuberculosis in a considerable percentage of them; whereas, the dust from rooms inhabited by healthy persons or dust of the streets does so only in an extremely small percentage. Flügge is probably right in thinking that the dust which is fine enough to remain for a long time in suspension in the air is usually free from living bacilli. It is in the coarser though still minute particles, those in which the bacilli are protected by an envelope of mucus, that the germs resist drying for considerable periods. These are carried only short distances by air currents. Such reports are those given by Straus, who, on examining the nasal secretions of twenty-nine healthy persons living in a hospital with consumptive patients, found tubercle bacilli in nine of them, must be accepted with some reserve, since we know that in the air there are bacilli which look and stain like tubercle bacilli and yet are totally different. It may be said that the danger of infection from slight contact with the tuberculous is not so great as it is considered by many, but that on this account it is all the more to be guarded against in the immediate neighborhood of consumptives. Those who are most liable to infection from this source are young children; the adult members of the family, the nurses, the fellow-workmen, and fellow-prisoners of persons suffering from the disease are relatively very less likely to become infected. In this connection, also, attention may be drawn to the fact that rooms which have been recently occupied by consumptives are not infrequently the means of producing infection (as has been clinically and experimentally demonstrated) from the deposition of tuberculous dust on furniture, walls, floors, etc. The danger is not apt to last beyond

three months. Flüge has drawn attention to the fact that in coughing, sneezing, etc., very fine particles of throat secretion containing bacilli are thrown out and carried by air currents many feet from the patient and remain suspended in the air for a considerable time. To encourage us, however, we now have a mass of facts which go to show that when the sputum is carefully looked after there is very little danger of infecting others except by close personal contact.

**Tuberculosis of Digestive Tract.**—Tuberculosis of the gums, cheeks, or tongue is rare. The tonsils and pharynx are somewhat more often involved. The stomach and esophagus are almost never attacked. The small intestines are rather frequently the seat of infection from bacilli swallowed with the food or dust-infected mucus. In a striking case four previously healthy children died within a short period of one another. Their nurse was found to have tuberculosis of the antrum of Highmore, with a fistulous opening into the mouth. She had the habit of putting the spoon with which she fed the children into her mouth so as to taste the food before it was given to them.

As already noted, the bacilli frequently pass through the mucous membrane of the gastro-intestinal canal to the lymph glands without leaving any lesions.

**Infection by Ingestion of Milk and Milk Products.**—Milk serves as a conveyor of infection, whether it be the milk of tuberculous mothers or the milk of tuberculous cows. In this case evidence of infection is usually shown in the mesenteric and cervical lymph nodes or generalized tuberculosis may be caused, while the intestinal walls are frequently not affected. Bacilli accompanied by fat pass much more readily through the intestinal mucous membrane or that of the tonsils and pharynx. The transmission of tubercle bacilli by the milk of tuberculous cows has been abundantly proved.

Formerly it was thought that in order to produce infection by milk there must be a local tuberculous affection of the udder; but it is now known that tubercle bacilli may be found in the milk in small numbers, when adjacent tissue is infected and when careful search fails to detect any udder disease. Schröder has shown that the feces are a very dangerous factor in the dissemination of tubercle bacilli. He compares feces in cattle to sputum in man, since the tubercle bacilli are swallowed by cattle and are to a great extent passed through the intestinal tract without destruction. He found that when milk from phthisical cows having healthy udders was obtained so as not to become infected by feces it was free from bacilli, but when obtained without special precautions it was frequently infected. The milk of every cow which has any well-developed internal tuberculous infection must therefore be considered as possibly containing tubercle bacilli. Rabinowitsch, Kempner, and Mohler also proved beyond question that not only the milk of tuberculous cattle, which showed no appreciable udder disease, but also those in which tuberculosis was only detected through tuberculin, frequently contained tubercle bacilli. Different observers have found tubercle bacilli in 10 to 30 per cent. of the samples of unheated

city milk. Butter may contain tubercle bacilli in higher percentages of samples examined. When we consider the prevalence of tuberculosis among cattle we can readily realize that, even if the bovine bacillus infects human beings with difficulty, there is danger to children when they are exposed to this source of infection. The milk from cattle suffering from udder tuberculosis usually contains a few hundred bacilli per cubic centimeter, but may contain many millions. It is also important to mention the fact that mixed milk from a herd, though tending to dilute the milk of cows excreting tubercle bacilli, may be badly infected from one cow, especially if this cow has udder disease.

Taking the abattoir statistics of various countries, we find that about 10 per cent. of the cattle slaughtered were tuberculous. A less probable source of infection by way of the intestines is the flesh of tuberculous cattle. Here the danger is considerably less, from the fact that meat is usually cooked, and also because the muscular tissues are seldom attacked. In view of the finding of the bovine type of bacilli in a considerable percentage of the cases of tuberculous children tested, the legislative control and inspection of cattle and milk is an absolute necessity. As a practical and simple method of preventing infection from suspected milk, sufficient heating of the milk used as food must commend itself to all. Human tubercle bacilli may be found in milk as instanced by feeding them one sample out of a series of city milks examined in the Research Laboratory by Hess.

**Method of Examining Milk for Tubercle Bacilli.**—Thirty cubic centimeters of milk are centrifuged at high speed and 10 c.c. of the lower milk and sediment collected. Four cubic centimeters of the cream is thinned with a little sterile water and injected into two guinea-pigs. The sediment is injected in amounts of 3 to 5 c.c. into other pigs. Larger amounts than this are apt to kill too many pigs from the associated bacteria. Subcutaneous injection is to be preferred. There are certain precautions that must be taken in drawing conclusions, as the different types of acid-fast "butter bacilli" may cause lesions, and their presence will be noted in smears made from these lesions. To avoid this source of error, two methods are resorted to. If cultures are made from the suspected lesions on glycerin agar, these bacilli usually develop in a few days, whereas tubercle bacilli do not. When one is ready to kill the pigs, 2 c.c. of old tuberculin should be injected into each pig late in the day. The following morning the tuberculous pigs will be dead or dying. Autopsies should be done on all to confirm the test. The milk should be as fresh as possible to prevent the growth of other bacteria.

**Bovine Infection in Man.**—Numerous investigations have been made on this point. To Ravenel probably belongs the credit of isolating the first bovine bacillus from a child. The following tables summarizing the results of a large series of cases give a fair idea of incidence of such infection. As will be seen, children are especially the ones infected, and usually the point of entry is clearly alimentary, as shown by the lesions. Cervical adenitis and abdominal tuberculosis are the most frequent types of infection. Generalized tuberculosis due to bovine

infection is less frequent. Bone and joint tuberculosis is usually of the human type. The meninges are less commonly affected by the bovine type than by the human type. Infection of adults is very uncommon; and, though cases of pulmonary tuberculosis due to the bovine type of bacillus have been reported, such cases are rare.

TABLE I.—TABULATION OF CASES REPORTED.<sup>1</sup>

Diagnosis.	Adults sixteen years and over.		Children five to sixteen years.		Children under five years.	
	Human	Bovine.	Human.	Bovine.	Human.	Bovine.
Pulmonary tuberculosis . . . . .	497	3	6	—	28	1
Tuberculous adenitis, axillary . . .	2	—	—	—	2	—
Tuberculous adenitis, cervical . . .	27	1	17	14	9	11
Abdominal tuberculosis . . . . .	15	4	7	8	9	11
Generalized tuberculosis, alimentary origin . . . . .	6	1	3	4	16	13
Generalized tuberculosis . . . . .	27	—	4	1	56	3
Generalized tuberculosis, including meninges, alimentary origin . . . . .	—	—	1	—	5	10
Generalized tuberculosis, including meninges . . . . .	4	—	10	—	51	—
Tubercular meningitis . . . . .	—	—	1	—	2	2
Tuberculosis of bones and joints . . .	31	1	31	3	20	—
Genito-urinary tuberculosis . . . . .	16	—	1	—	—	—
Tuberculosis of skin . . . . .	9	3	4	6	2	—
Miscellaneous cases:						
Tuberculosis of tonsils . . . . .	—	—	—	1	—	—
Tuberculosis of mouth and cervical nodes . . . . .	—	1	—	—	—	—
Tuberculous sinus or abscess . . . .	1	—	—	—	—	—
Sepsis, latent bacilli . . . . .	—	—	—	—	1	—
Totals . . . . .	635	14	85	37	201	51

Mixed or double infections:	10 cases.	
Pulmonary tuberculosis,	20 years.	Sputum, human and bovine types.
	27 years.	Sputum, human and bovine types.
Abdominal tuberculosis,	70 years.	Mesenteric nodes, human type.
		Retroperitoneal, human and bovine types.
Generalized tuberculosis	5½ years.	Spleen, human type.
(alimentary origin),		Mesenteric node, bovine type.
	18 years.	Lung, culture not obtained.
		Mesenteric nodes, human and bovine types.
	30 years.	Bronchial node, human type.
Generalized tuberculosis,	9 months.	Mesenteric nodes, human and bovine types.
including meninges		Bronchial nodes, bovine type.
(alimentary origin).		Mesenteric nodes, human type.
	4 years.	Meninges, human type.
Generalized tuberculosis,		Bronchial nodes, human type.
including meninges		Mesenteric nodes, bovine type.
	4 years.	Bronchial nodes } injected together.
including meninges.		Spleen } bovine and human types.
		Meninges, human type.
	4½ years.	Lung, human type.
		Mesenteric nodes, human type.
		Bronchial nodes, human and bovine types.
		Total cases, 1033.

<sup>1</sup> Summary of cases reported up to July, 1912, exclusive of cases examined at the Research Laboratory (see Table II). In contrast to the next table, the above contains a large percentage of selected cases of alimentary types of tuberculosis, many of which showed only slight lesions.

TABLE II.—THE RELATIVE PROPORTION OF HUMAN AND BOVINE TUBERCLE BACILLI INFECTIONS IN A LARGE SERIES OF UNSELECTED CASES<sup>1</sup> EXAMINED AT THE RESEARCH LABORATORY.

Diagnosis of cases examined.	Adults sixteen years and over.		Children five to sixteen years.		Children under five years.		Notes.
	Human.	Bovine.	Human.	Bovine.	Human.	Bovine.	
Pulmonary tuberculosis	281	—	8	—	7	—	Clinical diagnosis only known and therefore no positive details as to the extent of lesions elsewhere.
Tuberculous adenitis, inguinal and axillary	1	—	4	—	—	—	See next.
Tuberculous adenitis, cervical	9	—	19	8	6	13	In two cases cultures were from axillary nodes but the primary focus was cervical. Another case died shortly afterward with pulmonary tuberculosis.
Abdominal tuberculosis	1	—	1	1	1	3	Milk supply of one child subsequently examined. Tubercle bacilli isolated.
Generalized tuberculosis, alimentary origin	—	—	—	—	1	2	Only three cases given under this heading. Many of the cases in the following subdivisions showed marked intestinal lesions and some possibly were of alimentary origin.
Generalized tuberculosis	2	—	1	—	18	4	One bovine case had tuberculous osteomyelitis of the metatarsal bone.
Generalized tuberculosis including meninges	1	—	—	—	25	1	
Tuberculous meningitis	1	—	2	—	26	2	
Tuberculosis of bones and joints	1	—	10	—	7	—	No autopsy. Extent of lesions elsewhere unknown.
Genito-urinary tuberculosis	6	1	1	—	—	—	
Tuberculosis of skin	1	—	—	—	—	—	The adult bovine case was tuberculosis of kidney. Removal of kidney. Complete recovery.
Tuberculous abscess	1	—	—	—	—	—	
Totals	305	1	46	9	91	25	Possibly primary in bone.

Double infection in one case. Both types isolated. Generalized tuberculosis including meninges, thirteen months. Mesenteric nodes gave human type. Meningeal fluid gave bovine type.  
Total cases, 478.

A careful study of all the factors leads us to estimate that with the average raw milk supply about 10 per cent. of all deaths caused by tuberculosis in children under five is due to bovine infection.

<sup>1</sup> Unselected cases from the hospitals of New York City. For full resumé and discussion of results see Park and Krumwiede, Jour. Med. Res., vols. xxiii, xxv and xxvii.

The tables on pages 377 and 378 give a summary of the results obtained in the larger investigations so far carried out.

**Hypothesis of Transmissibility of Tubercle Bacilli to the Fetus.**—The transmission of tubercle bacilli from the mother to the fetus in animals occurs occasionally. With regard to tuberculosis in the human fetus the evidence is not so clear, though some 20 cases have been recorded of tuberculosis in newly born infants, and about a dozen cases of placental tuberculosis. As to the infection of the fetus from the paternal side, where the father has tuberculosis of the scrotum or seminal vessels, we have no reason to suppose that such can occur. There are, however, grounds for belief that infection in this way may take place from husband to wife.

**Attenuation.**—Tubercle bacilli when subjected to deleterious influences slowly decrease in virulence. Some strains lose their virulence when artificially cultivated for some time; some quickly, some slowly. Others retain their virulence indefinitely.

**Mixed Infection.**—In regions where tuberculous processes are on the surface, such as skin infections, and also when the infection itself is multiple, as in diseases of the glands of the neck from tonsillar absorption, there are frequently associated with the tubercle bacilli one or more other varieties of organisms. Those of most importance are the streptococcus, pneumococcus and influenza bacilli. While the influence of this secondary or mixed infection is not exactly known, yet both the local and systemic effects are undoubtedly unfavorable.

In regard to pulmonary tuberculosis, it should be remembered that Baldwin has shown that caseation, ulceration and cavity formation may be produced experimentally in the lungs of animals by the tubercle bacillus alone. Further, it has been found that fever, emaciation and other characteristic symptoms of tuberculosis may be caused by the tubercle bacillus independently of any other associated microorganisms. The preponderance of the evidence supports the view that the lesions and symptoms in this type of infection may be caused by the tubercle bacillus alone, but not infrequently secondary organisms may contribute to the more severe symptoms or may be largely responsible for the unfavorable progress of the disease.

**Individual Susceptibility.**—It was believed by many that in demonstrating that tuberculosis was due to a specific bacillus that its occurrence was sufficiently explained; but they left out another important factor in the production of disease—individual susceptibility. That this susceptibility, or “predisposition,” as it is improperly called, may be either inherited or acquired is now an accepted fact in medicine. It has even been thought that the physical signs and characters—the *phthisical habit*—which indicate this susceptibility can be externally recognized. At first the inherited susceptibility was considered more important than the acquired, but now much that was attributed to the former is known to be explained by the fact of living in an infected area. The acquired susceptibility may arise from faulty physical development or from depression, sickness, overwork, excessive use of alcohol, etc.

Unquestionably, vast differences exist in different individuals in the intensity of the tuberculous process in the lung. That this does not depend chiefly upon a difference in virulence of the infection is evident from the fact that individuals contracting tuberculosis from the same source are attacked with different severity, and that there is, as a rule, no great difference in degrees of virulence for animals in the tubercle bacilli obtained from different sources. The possibility of favorably influencing many cases of an existing tuberculosis by treatment also proves that, under natural conditions, there is a varying susceptibility to the disease. Clinical experience teaches, likewise, that good hygienic conditions, pure air, good food, freedom from care, etc., increase resistance and are aids to recovery. Animal experiments have shown that not only are there differences of susceptibility in various animal species, but also an individual susceptibility in the same species. The doctrine of individual susceptibility, therefore, is seen to be founded on fact, although the reasons for it are only partially understood. Certain infectious diseases reduce the resistance to tuberculous infection, the most noteworthy example being measles.

**Tuberculosis Immunity.**—As in other infectious diseases, various attempts have been made to produce an artificial immunity against tuberculosis, but the results so far have been disappointing. The expectation that the immunological mechanism operating in other bacterial diseases would also be found in tuberculosis has not been realized. As a consequence of recent researches, we are coming to the opinion that the usual manifestations of humoral immunity have a secondary importance in this disease and that to the tuberculous focus we must ascribe a greater role than has been given it in the past. Tuberculous infection presents certain phases which find no close analogy in other infectious processes. Several facts of fundamental importance have developed from the newer pathological and bacteriological investigations. An infection by the tubercle bacillus may take place, and foci of considerable size may develop without the production of sufficient disturbance to the bodily welfare to attract attention. Further, while a frank tuberculous infection may come to a clinical cure, yet the focus remains, and, even though completely walled off, may harbor virulent tubercle bacilli during the whole of the individual's normal life. It has now become established that a healed tuberculous focus present in the body, containing as it does bacillary protein, exerts an appreciable influence on the behavior of the body to subsequent infection. In the consideration of tuberculosis immunity, therefore, we must carefully bear in mind the distinction between tuberculous infection and tuberculous disease.

Koch was the first to discover that animals already infected with living bacilli reacted differently to an injection of tubercle bacilli than did normal animals. When virulent tubercle bacilli are injected into a healthy animal, tubercles develop at or near the point of inoculation and the infection then progresses and the tubercle bacilli are carried to the spleen, liver, lungs and the intermediate glands with the formation of foci

in these various organs. As a consequence, the animal finally succumbs to generalized tuberculosis. In the case, however, of an animal already tuberculous, the inoculation with virulent tubercle bacilli is followed by a quite different sequence of events. Shortly after such an inoculation there is a marked inflammatory reaction at the point of injection, followed by necrosis and possibly sloughing but with no advance of the infection beyond the point of inoculation. In other words, the animal suffers from a local toxic process but not from a true infection. Römer has given further details of this phenomenon. He found that if a small dose of tubercle bacilli be given at the second inoculation the local reaction soon subsides and healing results. But, on the other hand, if a large quantity of tubercle bacilli be injected the local process goes on to necrotic sloughing and the animal soon dies of cachexia. The tuberculous animal, therefore, when subjected to an injection of tubercle bacilli is not truly infected but suffers from an intoxication, the degree of which depends upon the quantity of tubercle bacilli introduced. Recent studies in tuberculosis emphasize the fundamental importance of Koch's and Römer's observations, and have forced a radical revision in our theories of tuberculous infection and immunity. By means of the various tuberculin reactions, particularly the intracutaneous of Mantoux and cutaneous of von Pirquet, and by the most careful postmortem studies on a great number of individuals, we now know that a large majority of human beings are infected with the tubercle bacillus and develop demonstrable tuberculous lesions before they reach the age of eighteen. A few children thus infected may succumb to the disease but by far the greater number show no conspicuous signs of the infection, their foci heal, and without the tuberculin reaction or the opportunity of examination after death, the presence of tuberculous infection would pass unsuspected and undetected.

These recent additions to our knowledge have given us a new and apparently a truer conception of the factors underlying tuberculous infection. It is quite probable that the majority of cases of pulmonary tuberculosis developing after the eighteenth year of age are caused not by infection from without but by the breaking down of an encapsulated focus acquired and healed during childhood. The greater morbidity during the age group of about twenty to thirty may be accounted for by the fact that this is usually the period of greatest physical and mental strain and stress and that it is this stress which breaks down the body's resistance to an infective focus, and which transforms a latent infection into active disease.

Although it is evident that the presence of a healed focus protects the individual to a certain degree against a subsequent infection by the tubercle bacillus, it is equally apparent that the exacerbations suffered by healed cases show that the protection acquired is at best only a relative one. We must therefore conclude that there exists in the human race no absolute immunity to tuberculosis, and that such increased resistance as we may possess is gained only at the hazard of an early infection.



**Immunization.**—Koch, reasoning from his observations on the ability of the tuberculous animal to resist subsequent infection, attempted to increase this resistance by the injection of certain modified products of the tubercle bacillus. To this end he prepared his original tuberculin, or "O. T." which, however, failed to fulfil his expectations. With the development of our modern theories of bacterial immunity the hope arose that protection might be actively acquired through the injection of the tubercle bacillus either attenuated or dead or of its products by way of stimulating the body to the production of specific antibodies; or, again, that protection might be passively conferred by the administration of the serum of animals made immune to the tubercle bacillus. From observations on experimental animals and from serological tests on individuals treated with the various tuberculins, we now know that the injection of antigens derived from the tubercle bacillus fails to call forth in the treated individual any marked response in the way of the production of demonstrable bodies and that serum therapy is of little or no avail.

It is true that in some cases resistance to the disease can be raised within certain limits, and that, in man, clinical cures may sometimes be effected by the aid of such therapeutic agents as tuberculin. Yet in these cases we find little or no evidence of the production of agglutinins, precipitins, lysins or complement-fixing antibodies. We are, consequently, forced to the conclusion that such immunity as has been established must have been the result of a physiological mechanism differing from the processes operating in other infectious diseases, and recent investigations, particularly those of Krause, point to the tuberculous focus as the prime factor in the production of such immunity as develops in tuberculous disease. Experimental analyses of the physiological action of tuberculin have shed new light on the problem. That it is the tuberculous focus which, in a large measure, determines the body's reactivity to tuberculin is shown by the fact that normal individuals, that is, individuals free from tuberculous infection, can tolerate the administration of a relatively large amount of tuberculin without exhibiting any appreciable symptoms. Tuberculin, therefore, is in itself non-toxic and the characteristic reaction following its application to the tuberculous body must be looked upon as an allergic phenomenon. Its manifestations are threefold: there is a local reaction at the point of application, a focal reaction at the site of infection and a general constitutional reaction. The local reaction appears as a more or less non-infective inflammation in the skin or on the mucous membranes, depending upon the point of application, and it is this phase of the reaction which has been so profitably utilized for the diagnosis of tuberculous infection (see "Tuberculin as a Diagnostic Aid," below). The focal reaction consists in vascular changes at the tuberculous lesion. There is a hyperemia with a consequent softening of the focus, and a liberation of antigenic or toxic focal products. Krause has shown that these products are inherently toxic and their liberation into the blood stream may occasion the fever, the malaise and the other symptoms character-

istic of tuberculin intoxication. It is likely that in addition to this the antigen of the tuberculin may react with such immune bodies as may be present in the infected, and therefore hypersensitive, body and thus contribute to the general reaction.

If the dose of tuberculin is not too great the hyperemia at the focus is transient and the body responds with an increased cellular activity at the focus, resulting, under favorable conditions, in a further proliferation of connective tissue and a more complete encapsulation of the tuberculous lesion. Should the dose of tuberculin, on the other hand, be excessive or the bodily resistance be deficient the focal reaction may lead to a softening and breaking down of the lesion with consequent bleeding, dissemination of the liberated tubercle bacilli and extension of the lesion. Inasmuch as the internal location of the lesion frequently precludes an opportunity for observing the focal reaction we must look to the general constitutional manifestations as an index of the degree of reaction produced. The goal of tuberculin treatment, therefore, is the stimulation of the focus by doses of tuberculin so graded and adjusted to the sensitiveness of the individual that the body's ability to respond to and control the focal hyperemia is not overtaxed. In this way cell proliferation, encapsulation and ultimate healing of the focus may be promoted.

**Tuberculin in Diagnosis and Therapy.**—From the foregoing it can be readily seen that by taking advantage of the allergic or hypersensitive state in tuberculosis we are able to use tuberculin both as a diagnostic aid and as a therapeutic agent.

**As a Diagnostic Aid.**—The presence of a tuberculous focus in the body, by some mechanism not yet wholly understood, sensitizes the skin and mucous membranes in such a way that the application of tuberculin to these tissues causes a more or less severe inflammatory reaction at the point of application. The preparation used is the Old Tuberculin ("O. T.") of Koch and the following represents the methods of application in their order of sensitiveness as described by Hamman.

1. Intracutaneous test of Mantoux.
2. Subcutaneous-local test ("Stich Reaktion").
3. Cutaneous test of von Pirquet.
4. Subcutaneous test.
5. Percutaneous test of Moro.
6. Conjunctival test of Calmette.

**The Intracutaneous Test of Mantoux.**—While more difficult to carry out than the cutaneous test, the intracutaneous is the more delicate, and is the most widely used. The test is carried out as follows: the inner surface of the forearm is cleansed with alcohol, then with ether, the skin is drawn taut, and the diluted tuberculin is injected from a tuberculin syringe (1 c.c. graduated in 50ths or 100ths) through a fine needle (a No. 26 preferably) which has been carefully inserted into, but not under, the skin. The total volume injected should be 0.1 c.c., and it is advisable to employ several dilutions of tuberculin in order to determine the degree of hypersensitiveness of the patient's skin. Usually four

simultaneous injections are given, using dilutions of old tuberculin of 1 to 10,000,000, 1 to 1,000,000, 1 to 100,000, and 1 to 10,000, representing respectively, 0.0000001, 0.000001, 0.00001 and 0.0001 gram of tuberculin. Separate sterile syringes should be used for each dilution and 0.1 c.c. of sterile salt solution should be similarly injected as a control. (See Methods of Diluting Tuberculins.) The reaction when positive appears in six to eight hours, reaches its maximum in twenty-four to forty-eight hours and generally subsides in six to ten days, and consists of infiltration, hyperemia, and, in severe reactions, vesiculation. The width of the area of infiltration and the degree of inflammation are noted. It not infrequently happens that a person fails to give a positive reaction at the first test yet shows the typical local manifestations when the test is repeated. This has given rise to the impression that an injection of tuberculin sensitizes the individual. Without further discussion it may be stated that, as far as we know, there is no skin sensitiveness without infection. The appearance of a positive reaction at the second injection may be looked upon as a true reaction, and it is likely that the first injection while eliciting no response in the skin has served to stimulate the latent hypersensitiveness of the cells.

**The Subcutaneous-local Test or "Stich Reaktion."**—The test is carried out as in the intracutaneous test with the difference that the needle is inserted into the subcutaneous tissue with the point of the needle directed toward the surface. The intracutaneous test is preferable.

**The Cutaneous Test of von Pirquet.**—This is performed as follows: The inner side of the forearm is cleansed with alcohol and ether and two small similar scarifications or scratches are made about three inches apart. Oozing of blood is to be avoided. On one spot or scratch a drop of tuberculin is placed and allowed to dry on the scarification. The tuberculin may be diluted to 25 per cent., if desired. The other spot is kept as a control. Both spots should be examined at the end of twelve, twenty-four, and thirty-six hours. A positive reaction appears after three to twenty-four hours and is usually at its height at thirty-six to forty-eight hours, and consists then of a slightly raised reddening of the skin somewhat circular in outline and usually about 10 mm. in diameter. Reactions under 5 mm. in diameter should be regarded as doubtful.

**The Subcutaneous Test.**—The object of this test was to elicit a constitutional reaction by the injection of old tuberculin under the skin. Owing to the severe reactions frequently obtained which resulted in harm to the patient it is now considered the better practice to abandon this test in favor of the Mantoux or von Pirquet methods.

**The Percutaneous Test of Moro.**—An ointment is made of equal parts of lanolin and tuberculin ("O. T."), and a small amount of it is rubbed into the skin on the chest. A positive reaction is shown by the development of reddening and papules.

**Ophthalmic Test of Calmette.**—Owing to the occurrence of serious accidents this test is little used. A drop of a 2 per cent. solution of tuberculin is applied to the lower conjunctival sac. The reaction is indicated by secretion and reddening of the inner canthus caruncle or lower lid, which may include the entire conjunctiva, with edema of the lids.

**Deductions and Limitations of the Tuberculin Test for Diagnosis.**—A positive reaction indicates the presence of a tuberculous focus but not necessarily of tuberculous disease. It tells nothing of the location, extent or activity of the lesion. Krause gives the following basis for interpreting the reaction: There is no cutaneous hypersensitiveness without a focus (tubercle); this hypersensitiveness appears coincident with the establishment of the focus; it diminishes with the healing of the focus; it varies directly with the intensity of the disease. It should be mentioned, further, that many advanced cases, particularly those in cachexia, fail to show any appreciable response to tuberculin tests, and also that measles diminishes hypersensitiveness.

**Tuberculin: Kinds and Preparation.**—There exist a large number of tuberculin preparations. The following, however, hold the highest favor and suffice for the needs of the diagnostician or practitioner:

**Tuberculin, Koch's "Old" (O. T.).**—Cultures of tubercle bacilli after six weeks' growth on 5 per cent. glycerin broth<sup>1</sup> are heated in the Arnold sterilizer to kill the bacilli, and filtered. This bacillus-free filtrate is evaporated to one-tenth its original bulk and after filtering through paper to remove the sediment it is ready for use. The tuberculin is therefore a heated 50 per cent. glycerin solution of the products of the bacilli in the culture fluid and such portions of the bacilli as go into solution. It is used chiefly for diagnostic purposes.

**Tuberculin B. F. (Bouillon Filtrate of Denys)** is made from a broth culture as above. The culture is not heated, but filtered first through paper and then through a Berkefeld filter to insure sterility. It differs from the original tuberculin in that it is neither heated nor concentrated, and contains only such constituents of the bacillus as are soluble or are developed in the culture medium during cultivation.

**Tuberculin B. E. (Bacillus Emulsion)** is produced by grinding up dried tubercle bacilli until no intact bacilli can be found on microscopic examination. This powder is then suspended in glycerin-water and heated to 60° C. for an hour or more to kill any viable tubercle bacilli. The proportion of bacillus to water is such that 1 c c. of fluid contains 5 mg. of bacillus substance. This preparation contains all the constituents of the tubercle bacillus in an unchanged state, and therefore corresponds to a bacterial vaccine.

The undiluted products keep for a long time if kept cool and protected from the light. The low dilutions keep for at least one month, but the high dilutions should not be used after two weeks.

**Method of Diluting Tuberculin.**—The following describes a method of dilution in terms of volume of finished tuberculin, and gives the weight equivalent. Although in the case of bouillon filtrate many use dilutions, taking into consideration the fact that the bouillon filtrate is not concentrated, or in the case of bacillus emulsion dilute according to the weight of solid substance contained, a uniform method for each seems advisable for reasons of simplicity.

If we consider the finished product in terms of cubic centimeters or grams regardless of the contents, the following is the method of dilution. Dilutions should be made with sterile saline to which 0.25 per cent. carbolic has been added.

<sup>1</sup> Broth should be made 1.5 per cent. acid to phenolphthalein.

Dilutions.	Amount of tuberculin.	Amount of diluent.	Content of tuberculin terms of finished product.	Using bacillus emulsion content of solid substance in each dilution will be:
A	1 c.c.	9 c.c.	1 c.c. = 0.1 c.c. or gm., or 100 c.mm. or mg.	1 c.c. = 0.5 mgm., or 0.5 mgm.
B	1 c.c. of dilution A	9 c.c.	1 c.c. = 0.01 c.c. or gm., or 10 c.mm. or mg.	1 c.c. = 0.05 mgm., or 0.05 mgm.
C	1 c.c. of dilution B	9 c.c.	1 c.c. = 0.001 c.c. or gm., or 1 c.mm. or mg.	1 c.c. = 0.005 mgm., or 0.005 mgm.
D	1 c.c. of dilution C	9 c.c.	1 c.c. = 0.0001 c.c. or gm., or 0.1 c.mm. or mgm.	1 c.c. = 0.0005 mgm., or 0.0005 mgm.
E	1 c.c. of dilution D	9 c.c.	1 c.c. = 0.00001 c.c. or gm., or 0.01 c.mm. or mgm.	1 c.c. = 0.00005 mgm., or 0.00005 mgm.
F	1 c.c. of dilution E	9 c.c.	1 c.c. = 0.000001 c.c. or gm., or 0.001 c.mm. or mgm.	1 c.c. = 0.000005 mgm., or 0.000005 mgm.

**Tuberculin Treatment.**—Tuberculin is not a cure for tuberculosis. It promotes healing and relapses are less frequent after its use. It should be used as an addition not as a substitute for the recognized methods of treatment. It is a two-edged weapon and should be employed only by those who have a thorough understanding of its possibilities for good, and unfortunately, for harm.<sup>1</sup>

**United States Government Directions for Inspecting Herds for Tuberculosis.**—"Inspection should be carried on while the herd is stabled. If it is necessary to stable animals under unusual conditions or among surroundings that make them uneasy and excited, the tuberculin test should be postponed until the cattle have become accustomed to the conditions they are subjected to, and then begin with a careful physical examination of each animal. This is essential, because in some severe cases of tuberculosis, on account of saturation with toxins, no reaction follows the injection of tuberculin, but experience has shown that these cases can be discovered by physical examination. This should include a careful examination of the udder and of the superficial lymphatic glands, and auscultation of the lungs.

"Each animal should be numbered or described in such a way that it can be recognized without difficulty. It is well to number the stalls with chalk and transfer these numbers to the temperature sheet, so that the temperature of each animal can be recorded in its appropriate place without danger of confusion. The following procedure has been used extensively and has given excellent results:

"(a) Take the temperature of each animal to be tested at least twice, at intervals of three hours, before tuberculin is injected.

"(b) Inject in the evening, preferably between the hours of six and nine, 0.4 c.c. of Koch's tuberculin previously diluted to 5 c.c. with sterile water. The injection should be made with a carefully sterilized hypodermic syringe. The most convenient point for injection is back of the left scapula. Prior to the injection the skin should be washed carefully with 5 per cent. solution of carbolic acid or other antiseptic.

"(c) The temperature should be taken nine hours after the injection, and temperature measurements repeated at regular intervals of two or three hours until the sixteenth (eighteenth)<sup>2</sup> hour after the injection.

"(d) When there is no elevation of temperature at this time the examination may be discontinued; but if the temperature shows an upward tendency, meas-

<sup>1</sup> For details of tuberculin treatment see Haman and Wollman, "Tuberculin in Diagnosis and Treatment," Appleton, New York, 1912.

<sup>2</sup> The directions allow temperatures to be stopped the sixteenth hour, but even when there is no reaction at all it is much safer to always take temperatures for eighteen hours. We have found now and then a tuberculous cow that reacted on the eighteenth hour for the first time.

urements must be continued until a distinct reaction is recognized or until the temperature begins to fall.

"(e) If a cow is in a febrile condition tuberculin should not be used, because it would be impossible to determine whether, if a rise of temperature occurred, it was due to the tuberculin or to some transitory illness.

"(f) Cows should not be tested within a few days before or after calving, for experience has shown that the result at these times may be misleading.

"(g) In old, emaciated animals and in retests, use twice the usual dose of tuberculin, for these animals are less sensitive.

"(h) Condemned cattle must be removed from the herd and kept away from those that are healthy.

"(i) In making postmortems the carcasses should be thoroughly inspected, and all the organs should be examined."

**Antituberculous Serum.**—Every conceivable way of obtaining the true products of the tubercle bacilli has been tried, so as to cause the injected animals to produce antibodies both antitoxic and bactericidal. In spite of much conflicting testimony, it is probably safe to assert that no serums now obtainable have any great value.

**Prophylaxis.**—All energies should be directed to the prevention of tuberculosis, not only by the enforcement of proper sanitary regulation as regards the care of sputum, milk, meat, disinfection, etc., but also by continued experimental work and by the establishment of free consumptive hospitals, and by efforts to improve the character of the food, dwellings, and conditions of the people in general, we should endeavor to build up the individual resistance to the disease. It may be years before the public are sufficiently educated to coöperate with the sanitary authorities in adopting the necessary hygienic measures to stamp out tuberculosis entirely; but, judging from the results which have already been obtained in reducing the mortality from this dread disease, we have reason to believe that in time it can be completely controlled.

Among the numerous medical agents that have been tried without avail to protect animals against the action of the tubercle bacillus may be mentioned tannin, menthol, sulphuretted hydrogen, mercuric chloride, creosote, creolin, phenol, arsenic, eucalyptol, etc.

**Agglutination and Complement-fixation Reaction for Diagnosis.**—The results obtained with the agglutination reaction by various observers have been very conflicting. At present the test cannot be advised as useful in diagnosis, as the sera of cases suffering from tuberculosis frequently fail to give a reaction, while the sera from those having no detectable tuberculosis frequently cause a good reaction. A reaction in dilutions of 1 to 10 or 1 to 15 is considered a positive test (For Complement-fixation see p. 200.)

**The Tubercle Bacillus of Domestic Animals and its Relation to Human Tuberculosis.**—Among the domestic animals tuberculosis is most common in cattle. On account of the milk which they provide for our use, and which is likely to contain bacilli, the relation of these to human tuberculosis is a matter of extreme importance.

The chief seat of the lesions is apt to be the lymphatic nodes or lungs, and with them the pleura; less often the abdominal organs and the udder are affected. In pigs the abdominal organs are more often

involved, then the lungs and lymphatic glands. In sheep, horses and goats tuberculosis is rare.

**Differences between Tubercle Bacilli of Human and Bovine Type.**—As has been already noted in the tables given of the incidence of bovine and human infection, it is possible to tell in any case the type of infection. The essential differences are in cultural characteristics and in virulence for rabbits and calves.

**Cultural Differences.**—The bovine bacillus grows very poorly when isolated, the human bacillus very freely. This is noted on plain egg, but to a less extent than on glycerin egg. The glycerin restrains or adds little to the growth of bovine bacilli, but increases markedly the amount of growth of the human bacillus. In fact, primary cultures on glycerin egg of bovine material commonly fail to grow. This difference is very noticeable in the first few generations and is sufficient in the great majority of instances for differentiation to one who has had some experience with such cultures. Further, the majority of human strains can be transplanted to glycerin potato or glycerin broth and give vigorous growth in the first few generations, whereas the bovine bacillus fails to grow or growth is very slight. After further cultivation the bovine bacillus gradually increases its amount of growth until it is indistinguishable from the human type. This increase in luxuriance may be rapid or very slow.

**Rabbit Virulence.**—The bovine bacillus is exceedingly virulent for rabbits by any method of inoculation; the human bacillus only slightly so. The best method of differentiation is by intravenous inoculation. A small amount of culture is weighed after the moisture has been extracted with filter paper, and a suspension made in normal saline and diluted so that 1 c.c. = 0.01 mg. of culture; this amount is then injected into the ear vein of a rabbit. If the rabbit survives for from forty to fifty days, and on autopsy shows only lesions in the lungs or kidneys or both, the strain is of the human type. With the bovine type of bacillus the rabbit will die in the majority of instances before or about this time, if not it may be killed. On autopsy a progressive generalized tuberculosis will be found. The lesions in the lungs will be very marked, the tubercles having become confluent with caseous centres. The liver or spleen or both will be peppered with tubercles. Tubercles will be present in the great majority of cases in the superficial lymph nodes and also in those of abdomen and thorax. There may be tubercles on the heart, in the rib marrow, or over the peritoneum.

These two differences alone are sufficient to differentiate in every case the type of bacillus. It must be insisted upon again that the cultural characteristics be observed in the early generation and, further, that the virulence be tested in early generations. In case the bovine culture does not afford sufficient material for weighing, a suspension can be made and compared with a weighed suspension.

**Virulence for Calves.**—In proving the non-identity of the two bacilli, calf experiments were resorted to. This was necessary as the supposed bovine cultures from children would have to be virulent for calves to

the same extent as cultures from bovine material. The commonly used method was the subcutaneous inoculation in the side of the neck with 50 mg. of culture. The human type of bacillus caused only a local lesion or at most a spreading to the nearest lymph node. The bovine bacillus, on the other hand, caused a generalized tuberculosis which was or was not fatal. Sufficient data has been accumulated to make this test practically unnecessary for the determination of type.

**Differences in Morphology.**—The bovine bacillus tends to be shorter, thicker, and solidly stained; the human type tends to be longer, slimmer, usually bent, and shows beading and irregularities in staining. We have found this difference most marked on glycerin egg, slight or imperceptible on other media.

Besides the above differences Theobald Smith made the interesting discovery that the production of acid differed with the two types when grown on glycerin broth. The bovine type renders the bouillon less and less acid; this may even progress until the medium becomes slightly alkaline to phenolphthalein. The human type causes a preliminary fall in the acidity; as growth progresses the acidity is then gradually increased, and may exceed the original acidity of the broth used. This difference is evident in tuberculin made from the two types of bacilli. The bovine tuberculin is alkaline or very slightly acid while human tuberculin is markedly acid. The change is only noticed when glycerin is used in the media. The work of more recent investigators would seem to show that this difference, like all differences between the types, is purely quantitative, and that different strains vary in their reactions and give intermediate reactions between these two extremes.

**Bird (Avian) Tuberculosis.**—Tuberculosis is very common among fowl. The bacillus grows easily and freely on glycerin media. It tends to form a moist or even slimy growth, and commonly produces an orange pigment. It is able to grow at a higher temperature than mammalian tubercle bacilli, the latter failing to grow above 41° C., the former growing at even higher temperatures. Guinea-pigs are less susceptible to inoculation with avian tubercle bacilli, and the virulence for these animals is usually quickly lost. Rabbits are much more susceptible. Rats and mice are spontaneously infected with avian tubercle bacilli and are supposed to be an important factor in spreading the disease. Birds are refractory, with few exceptions, to infection with the mammalian tubercle bacillus. Parrots, however, are susceptible to infection with all three types and commonly have spontaneous tuberculosis caused by the human type of bacillus.

**Stability of the Different Types of Bacilli.**—The fact that the agglutination reactions and the tuberculin reactions of the different types is similar shows their close relationship. This has led to the endeavor to change one type into the other. This is usually attempted by passage through animals. The results have been peculiar. Some cultures have been passed through a series of calves without any change except for a moderate increase in virulence. Other cultures seem to have completely changed their type. We believe that this is not a change of



type, but an additional bovine infection. Strong negative evidence found is the fact that the bovine bacillus when infecting man loses none of its characteristics, though present in the human body for years.

**Tuberculosis of Cold-blooded Animals.**—The bacilli of this group are of interest mainly because of the claims of Friedmann that immunity against the tubercle bacillus is produced by their injection, and the publicity given to his claims that vaccines of these organisms have a curative influence in tuberculosis. The results of the use of the so-called Friedmann vaccine have, however, been unsatisfactory. A similar attempt to employ the cold-blooded types for immunization is the use in cattle as advised by Klimmer.

These types have been isolated from spontaneous, tuberculous-like lesions of frogs, lizards, turtles, fishes, etc. They have little resemblance to the mammalian types of tubercle bacilli other than their acid-fastness. They grow rapidly and luxuriantly on ordinary media and their optimum of growth is at 20° to 30° C., higher temperatures inhibiting their growth. They are not pathogenic for warm-blooded animals, although toxic symptoms or limited lesions may be produced by the injection of large doses as with most other non-pathogenic acid-fasts. Tuberculins prepared from them are not toxic for tuberculous mammalia except in large doses, so that a specific reaction can be excluded. It is possible but very improbable that they produce some antibodies which would protect against mammalian bacilli to a limited extent.

**Methods of Examination for Tubercle Bacilli.**—One of the most important results of the discovery of the tubercle bacillus relates to the practical diagnosis of tuberculosis. The staining peculiarities of this bacillus render it possible by the bacteriological examination of microscopic preparations to make an almost positive diagnosis in the majority of cases. A still more certain test in doubtful cases is the subcutaneous or intraperitoneal injection of guinea-pigs, which permits of the determination of the presence of numbers of bacilli, so small as to escape detection by microscopic examination. For the animal test, however, time is required—at least three weeks, and, if the bacilli present are very few in number, at least six weeks—before any positive conclusion can be reached, for when only a few bacilli are present tuberculosis develops slowly in animals. In disinfection experiments where many dead bacilli are injected, care must be taken to exclude the local effect of dead bacilli. In doubtful cases a second guinea-pig should be injected with material from the first.

**Microscopic Examination of Sputum for the Presence of Tubercle Bacilli.**—1. **Collection of Material.**—The sputum should be collected in a clean bottle (two-ounce) with a wide mouth and a water-tight stopper, and the bottle labelled with the name of the patient or with some other distinguishing mark. The expectoration discharged in the morning is to be preferred, especially in recent cases, and the material should be coughed up from the lungs. Care should be taken that the contents of the stomach, nasopharyngeal mucus, etc., are not discharged during the act of expectoration and collected instead of pulmonary

sputum. If the expectoration be scanty the entire amount discharged in twenty-four hours should be collected. In pulmonary tuberculosis the purulent, cheesy, and mucopurulent sputum usually contains bacilli; while pure mucus, blood, and saliva, as a rule, do not. When hemorrhage has occurred, if possible some purulent, cheesy, or mucopurulent sputum should be collected for examination. The sputum should not be kept any longer than necessary before examination, for, though a slight delay or even until putrefaction begins, does not vitiate the results so far as the examination for tubercle bacilli is concerned, it almost destroys any proper investigation of the mixed infection present; it is best, therefore, to examine it in as fresh a condition as possible, and it should be kept on ice until examined if cultures are to be made.

**2. Methods of Examination.**—*Examination for Tubercle Bacilli.*—Pour the specimen into a clean, shallow vessel, having a blackened bottom—a Petri dish placed upon a sheet of dull black paper answers the purpose—and select from the sputum some of the true expectoration, containing, if possible, one of the small white or yellowish-white cheesy-looking masses or “balls.” From this make rather thick cover-glass or slide “smears” in the usual way. In doubtful cases a number of these coarse or fine particles should be placed on the slide. The material being thick should be evenly spread and very thoroughly dried in the air before heating. (For methods of staining see pp. 77 and 80.)

Occasionally one is able to demonstrate the presence of tubercle bacilli with the Hermann stain where Ziehl's carbol-fuchsin gives negative results. At least two smears should be made and examined, if possible, before a negative report is given. Many of the incipient cases will require several examinations before bacilli are found. Some will remain consistently negative. It must be remembered that lesions may exist and that without ulceration the bacilli do not find their way into the sputum.

*Methods for Concentrating the Bacilli.*—Uhlenhuth advises the use of antiformin. This is a patented preparation consisting of a mixture of sodium hydroxide and sodium hypochlorite solution. If this is mixed with sputum so that the total strength is about 15 per cent. of antiformin, the sputum quickly becomes fluid. This should be thinned with water or alcohol to help reduce the specific gravity of the mixture and centrifuged. The sediment is then mixed with water and recentrifuged, and the washed sediment used for smears. Besides the dissolving action, antiformin kills most of the bacteria in the sputum, but not the tubercle bacilli, though they are slowly affected, so that sediment may be used for cultural purposes or injection into guinea-pigs.

A comparison of the above methods made by us gave the following results: Of twenty-eight sputa negative with carbol-fuchsin, two showed bacilli after a few minutes, search with the crystal violet stain. On restaining with carbol-fuchsin and giving only a light counter-stain with methylene blue the negative slides were also positive. Of the remaining twenty-six, four (15 per cent.) were quickly positive in the antiformin

sediment when stained with crystal violet, whereas only three were positive with carbol-fuchsin and only after restaining as above. It is advisable, therefore, in using carbol-fuchsin to have only a light counter-stain to make the method most efficient, and control the results with crystal violet if negative.

In place of sedimenting the bacilli, the dissolved sputum may be shaken up with a hydrocarbon. When the hydrocarbon separates out from the sputum the waxy tubercle bacilli adhere to it and are collected in a layer between the dissolved sputum and the hydrocarbon. If the hydrocarbon is heavy, chloroform, they are carried down, if light, ligroin, they are carried up.

Kinyoun has modified the original ligroin method as given below. We have had very satisfactory results with its use. As a routine method it saves time and gives a high percentage of positive results.

Bottles of about 15 c.c. capacity containing about 2 c.c. of a 1 per cent. solution of cresol are used for collection. The cresol is added to limit decomposition of the sputum if its transit to the laboratory is delayed. When received 1 c.c. of ligroin (specific gravity not less than 0.715 or more than 0.72) is added and the bottle filled with an alkaline solution of hypochlorite of lime. If the bottle is full, about one-third must be poured out to allow for the addition of the solution. The hypochlorite solution is prepared as follows: Three packages of chlorinated lime are weighed, and for each 90 grams, 65 grams of sodium carbonate are taken. The lime is mixed with 500 to 600 c.c. of water and the carbonate is dissolved in 1500 c.c. of water by boiling. The carbonate is then added to the lime and thoroughly mixed. After standing twelve to twenty-four hours the solution is filtered off. The amount of available chlorine is estimated and the solution diluted so that the chlorine is 0.56 per cent. Then 7.5 grams of caustic soda are added to each 100 c.c. of the filtrate. The solution should be kept cool and in the dark. Fresh lots should be prepared about every three months.

After the addition of the ligroin and the solution of chlorine the bottles of sputum are placed in a shaking machine and thoroughly shaken for five to ten minutes. The bottles can then be allowed to stand until the ligroin rises, which takes several hours, or this can be hastened, placing the bottles in a centrifuge, with special cups to accommodate the bottles, and run for about ten minutes at moderate speed.

When the ligroin rises to the top a soapy layer develops at the point of contact with the fluidified sputum and the tubercle bacilli are collected in this layer. The soapy layer is taken up with a platinum loop and smears made on glass slides fixed by heat and stained. Individual slides must be used and the slides must be stained separately or error will result, as the bacilli are not firmly fixed to the slides.

All the antiformin methods must be used with caution, as it is easy to see how error can creep in from contamination with other acid-fast bacilli. (See also the Petroff method given above.)

**Detection of Tubercle Bacilli in Urine and Feces, etc.**—The catheterized urine is centrifuged. If little sediment appears, the upper portion of the fluid is removed and more urine added and again centrifuged. If the urine is rich in salts of uric acid, the same may be diminished by carefully warming the urine before treating it. If too alkaline add a little acetic acid. A possible source of error is the presence of smegma bacilli.

The feces are examined for any purulent or mucous particles. If none are found, larger masses of feces are removed and then the rest diluted and centrifugalized. The antiformin methods are a great aid in the examination of feces. The examiner must remember that bacilli swallowed with the sputum may appear in the feces.

In examining cerebrospinal fluid for tubercle bacilli it must be remembered that the majority of the bacilli are entangled in the delicate clot that forms. Whenever possible the fluid after withdrawal should be allowed to stand until this filmy clot develops, which is then fished out and examined. If this is impossible the fluid should be centrifuged and the sediment stained. This is also the case in other serous fluids, but in ascitic or pleuritic fluid they are usually very few in number. (For sputum washing see p. 136.)

**Inoculation of Animals.**—The inoculation of suspected material into guinea-pigs produces tuberculosis; even if the number of bacilli is very small. When no bacilli can be detected by microscopic examination this can be done for diagnostic purposes. The material should be injected subcutaneously as already described.

**Cultivation.**—This requires so much time that it is not generally used except in important investigations upon the nature of the tubercle bacilli. The special methods have already been given.

## CHAPTER XXVIII.

### OTHER ACID-FAST BACILLI: BACILLUS OF LEPROSY, BACILLUS OF RAT LEPROSY, BACILLUS OF JOHNE'S DISEASE IN CATTLE, AND THE GROUP OF NON-PATHOGENIC ACID-FAST BACILLI.

#### LEPROSY BACILLUS—*B. LEPRÆ*.

THE bacillus of leprosy was discovered by Hansen and Neisser (1879) in the leprous tubercles of persons afflicted with the disease. This discovery was confirmed by many subsequent observers.

**Morphology (in Tissues).**—Small, slender rods resembling the tubercle bacilli in form, but somewhat shorter and not so frequently curved. The rods have pointed ends, and in stained preparations unstained spaces, similar to those observed in the tubercle bacillus, are seen. They *stain* readily with the aniline colors and also by Gram's method. Although differing slightly from the tubercle bacillus in the ease with which they take up the ordinary aniline dyes, they behave like tubercle bacilli in retaining their color when subsequently treated with strong solutions of the mineral acids and alcohol. The difference in staining characteristics is too slight to be relied upon for diagnostic purposes (see Plate VI).

**Bacilli Isolated from Leprous Lesions.**—No acid-fast organism was grown from leprous lesions until Clegg reported, in 1908, that he had been able to cultivate an acid-fast bacillus by growing it in symbiosis with ameba and cholera. Since then Duval, Kedrowski, Twort, and many others have reported the finding of various more or less acid-fast organisms in leprous lesions. These organisms may be grouped as follows:<sup>1</sup>

**Bacilli of the Diphtheroid Type.**—In serum media the colonies are yellowish white and develop best at 37° C., although a slight growth occurs at room temperature. When growth occurs on broth, the medium remains clear, and a pellicle is produced. The morphology is variable. They are either solidly stained or irregularly stained like other types of diphtheroids. They are Gram-positive and may show some resistance to decolorization after staining with carbol-fuchsin, especially the metachromatic granules.

*Pathogenicity*, none or questionable.

**Acid-fast Chromogenic Bacilli.**—This type of bacillus is difficult to isolate but after isolation grows freely at both 37° and 20° C. on

<sup>1</sup> The results of all the investigations cannot be given. For a fuller discussion and bibliography see the excellent *resumé* of Wolbach and Honeij: Jour. Med. Research, 1914, xxix, 367.

most of the ordinary media. The growth is luxuriant, moist, and a yellow to deep orange color develops. The individual bacilli vary in morphology from coccoid to filamentous bacilli, some showing metachromatic granules, others showing clear areas. They are acid-fast but less so than the tubercle bacillus.

*Pathogenicity.*—Lesions similar to leprosy produced in Japanese dancing mice and in monkeys.

**Anaërobic Bacilli.**—In this place it is sufficient to state that such organisms have been isolated.

**Acid-fast Non-chromogenic Bacilli.**—These types are characterized by their feeble, slow growth on artificial media, and growth only takes place at 37° C., and then only on special media.

Morphologically they vary from plump to long, slender bacilli, often beaded or bipolar in appearance.

*Pathogenicity*, none.

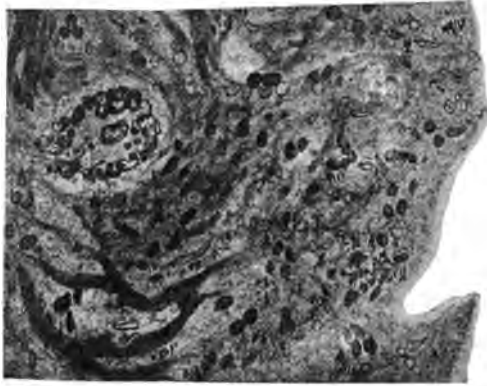


FIG. 141.—Leprosy bacilli in nodule. (Kolle and Wassermann.)

What conclusion is to be drawn from such variable results is difficult to say. Has the diphtheroid bacillus any relationship to the more acid-fast types? This is a possibility when one considers that under certain circumstances it shows some resistance to decolorization. Then, too, the leprosy bacilli in tissue may decolorize easily, although they are abundant, as shown by staining with polychrome methylene blue. On the other hand, the repeated isolation of diphtheroids from the lymph nodes in other conditions raises a strong element of doubt as to the etiological significance of this organism. A careful comparison of the diphtheroids from various conditions, especially their resistance to decolorization, might give us some help.

As to the chromogenic acid-fasts, the character of their growth, viz., the luxuriant growth upon ordinary media at low temperatures is not that of a highly specialized parasite. As to the apparently successful animal inoculations it must be remembered that lesions have been produced by acid-fast bacilli, known to be non-pathogenic. Why such

bacilli should be frequently isolated from leprous lesions is still to be explained.

The non-pigmented types are more consistent with our idea of what the bacillus should be but whether they are actually the etiological organism remains to be seen.

The serum reactions, such as agglutination and complement-fixation, have added no evidence as to the etiological significance of any one of the bacilli isolated. Each of the bacilli mentioned has been agglutinated by sera of lepers. The complement-fixation reactions await a successful specific test for the individual acid-fast organisms.

**Pathogenesis.**—Numerous inoculation experiments have been made on animals with portions of leprous tubercles, but there is no conclusive evidence that leprosy can be transmitted to the lower animals by inoculation. The inference that this bacillus bears an etiological relation to the disease with which it is associated is based chiefly upon the demonstration of its constant presence in leprous tissues.

The bacilli are found in all the diseased parts, and usually in large numbers, especially in tubercles on the skin, in the conjunctiva and cornea, the mucous membranes of the mouth, gums, and larynx, and in the interstitial processes of the nerves, testicles, spleen, liver, and kidneys. The rods lie almost exclusively within the peculiar round or oval cells of the granulation tissues which compose the leprous tubercles, either irregularly scattered or arranged parallel to one another. In old centres of infection the leprosy cells containing the bacilli are larger and often polynuclear. Giant cells, such as are found in tuberculosis, are claimed to have been observed by a few investigators (Boinet and Borrel). In the interior of the skin tubercles, the hair follicles, sebaceous and sweat glands are often attacked, and bacilli have sometimes been found in these (Unna, etc.). Quite young eruptions often show a few bacilli. A true caseation of the tubercles does not occur, but ulceration results. During acute exacerbations with development of new lesions bacilli have been observed in the blood.

In the anesthetic forms of leprosy the bacilli are found most commonly in the nerves and less frequently in the skin. They have been demonstrated in the sympathetic nervous system, in the spinal cord, and in the brain. The *Bacillus lepræ* occurs also in the blood, partly free and partly within the leukocytes, especially during the febrile stage which precedes the breaking out of fresh tubercles (Walters and Doutrelept). The bacilli have also been found in the intestines, in the lungs, and in the sputum, but not in the urine.

With regard to the question of the direct inheritance of the disease from the mother to the unborn child there is considerable difference of opinion. Some cases have been reported, however, in which a direct transmission of the bacillus during intra-uterine life seems to be the only or most plausible explanation of the infection. At the same time, we have no positive experimental evidence to prove that such an infection does take place. Although many attempts have been made to infect healthy individuals with material containing the bacilli of leprosy,

the results are not conclusive. Even the experiments made by Arning, who successfully infected a condemned criminal in the Sandwich Islands with fresh leprous tubercles, and which have been regarded as positive evidence of the transmissibility of the disease in this way, are by no means conclusive; for, according to Swift, the man had other opportunities for becoming infected. The negative results, together with the fact that infection does not more frequently occur in persons exposed to the disease, may possibly be explained by the assumption that the bacilli contained in the tuberculous tissues are mostly dead, or much more probably that an individual susceptibility to the disease is requisite for its production.

The widespread idea, before the discovery of the leprosy bacillus, that the disease was associated with the constant eating of dried fish or a certain kind of food, has now been entirely abandoned.

The relation of leprosy to tuberculosis is sufficiently evident from their great similarity in many respects. This is rendered still more remarkable by the fact that leprosy reacts, both locally and generally, to an injection of tuberculin in the same manner as tuberculosis, but to a somewhat less extent.

**Rat Leprosy.**—The interest in this disease lies in the fact that diphtheroid and chromogenic acid-fast bacilli similar to those described above have been isolated from leprous rats.

**Bacillus of John's Disease, Chronic Enteritis or Paratubercular Dysentery of Cattle.**—This disease is comparatively common in this country and is characterized by chronic diarrhea and emaciation, commonly leading to death. The intestinal mucosa is thickened, and the lesions are not limited. Tubercle formation and necrosis are absent. The bacilli are present in the lesions in enormous numbers. Twort succeeded in cultivating the organism and his work was verified by Holth and Meyer. A tuberculin made from this organism will probably be of diagnostic value. Animals having this disease do not react to the ordinary tuberculin test but do react to large doses of tuberculin made from the avian type of tubercle bacillus. The bacillus is not pathogenic for guinea-pigs or rabbits, although local abscesses may be produced.

**Non-pathogenic Acid-fast Bacilli.**—These have no importance further than historical interest and the fact that they may be present in materials suspected of containing tubercle bacilli and thus lead to error. They vary widely in their acid-fastness, especially when artificially cultivated. Differential staining methods have been devised to separate them from the tubercle bacillus and although in a general way the decolorization by prolonged action of acid and alcohol is presumptive evidence against suspected bacilli being tubercle bacilli, it is an unsafe procedure. Tubercle bacilli vary in their acid-fastness but the non-pathogenic types vary even more widely, some being extremely resistant to decolorization. Many of the non-pathogenic types grow rapidly at low temperatures and in cultures can thus be quickly differentiated from tubercle bacilli.

They can be separated from tubercle bacilli by inoculating animals in which no progressive lesions will develop, although limited lesions may be produced if they are injected in large numbers. The guinea-pig may be injected with 2 c.c. of tuberculin and if infected with tuberculosis will die, but if by other acid-fast bacilli, will show little or no reaction. If a second group of guinea-pigs are inoculated with a small amount of the infected tissue from the inoculated pigs there will develop progressive tuberculosis if the doubtful bacilli were tubercle bacilli, and practically no lesions if they were grass bacilli. Cultures from the lesions may also be an aid in differentiation.



**Bacillus of Lustgarten.**—This bacillus was found by Lustgarten in 1884 in syphilitic lesions or ulcers. It is undoubtedly a saprophyte. It is very similar morphologically to the smegma bacillus and may be identical with it. It is of historical interest only.

**Smegma Bacillus.**—This bacillus is present in smegma from the prepuce or vulva. Its only interest is the danger of mistaking it for tubercle bacilli in the examination of urine, especially if the latter be carelessly collected.

**Timothy and Other Grass Bacilli.**—On various grasses, in cow manure, in butter and in milk there have been found bacilli with varying degrees of acid-fastness. Similar bacilli have also been demonstrated in water. They make the direct microscopic examination of such material for tubercle bacilli of little value and the nature of any acid-fast organisms so found must be determined by animal inoculations.

## CHAPTER XXIX.

### GLANDERS BACILLUS (BACILLUS MALLEI). B. ABORTUS (BANG).

#### GLANDERS BACILLUS.

THE *Bacillus mallei* was discovered and proved to be the cause of glanders by several bacteriologists at almost the same time (1882). Bouchard, Capitan and Charin obtained it in mixed cultures, while it was first accurately studied in pure culture by Löffler and Schütz. It is present in recently formed nodules in animals affected with glanders, in the nasal discharge, in pus from the specific ulcers, etc., and occasionally in the blood.

**Morphology.**—Small bacilli with rounded or pointed ends, from nutrient agar cultures,  $0.25\mu$  to  $0.5\mu$  broad and from  $1.5\mu$  to  $5\mu$  long; usually single, but sometimes united in pairs, or growing out to long filaments, especially in potato cultures. The bacilli frequently break up into short almost coccus-like elements (Fig. 142).

**Staining.**—The *bacillus mallei* stains with difficulty with the aniline colors, best when the aqueous solutions of these dyes are made feebly alkaline; it is decolorized by Gram's method. This bacillus presents the peculiarity of losing very quickly in decolorizing solutions the color imparted to it by the aniline staining solution. For this reason it is difficult to stain in sections. Löffler recommends his alkaline methylene-blue solution for staining sections, and for decolorizing, a mixture containing 10 c.c. of distilled water, 2 drops of strong sulphuric acid, and 1 drop of a 5 per cent. solution of oxalic acid; thin sections to be left in this acid solution for five seconds.

**Biology.**—A non-motile bacillus, whose molecular movements are so active that they have often been taken for motility. It is aerobic, but moderate multiplication occurs in the depths of culture media. Grows well on culture media at  $37^{\circ}$  C. Development takes place



FIG. 142.—Glanders bacilli. Agar culture.  
× 1100 diameters.

slowly at 22° C. and ceases at 43° C. The bacillus does not form spores. Exposure for ten minutes to a temperature of 55° C., or for five minutes to a 3 to 5 per cent. solution of carbolic acid, or for two minutes to a 1 to 5000 solution of mercuric chloride destroys its vitality. As a rule the bacilli do not grow after having been preserved in a desiccated condition for a week or two; in distilled water they may live twenty-five days. It is doubtful whether the glanders bacillus finds conditions in nature favorable to a saprophytic existence.

A solution of chlorinated lime, containing 1 part of free chlorine per 1000, is useful as a disinfectant of stables and utensils; it kills the bacillus in from one to two minutes. Strong sodium carbonate solution (washing soda) is also useful.

**Cultivation.**—(For obtaining pure cultures see page 401.)—It grows well at 37° C. on *glycerin-veal agar*, an acidity of 1.5 to 2.5 (phenolphthalein) being the most favorable. Upon this medium, at the end of twenty-four to forty-eight hours, whitish, transparent colonies are developed, which in six or seven days may attain a diameter of 7 or 8 mm. On *blood serum* a moist, opaque, slimy layer develops, which is of a yellowish-brown tinge. The growth on cooked *potato*, that is, sterilized, is especially characteristic. At the end of twenty-four to thirty-six hours at 37° C. a moist, yellow, transparent layer develops; this later becomes deeper in color, and finally takes on a reddish-brown color, while the potato about it acquires a greenish-yellow tint. In *bouillon* the bacillus causes diffuse clouding sometimes with a pellicle, ultimately with the formation of a more or less ropy, tenacious sediment. The broth should also be as acid in reaction as the glycerin-veal agar above. The addition of potato juice to either of these media is most favorable to the growth of the organism. *Milk* is coagulated with the production of acid.

**Pathogenicity.**—The bacillus of glanders is pathogenic for a number of animals. Among those which are most susceptible are horses, asses, guinea-pigs, cats, dogs, ferrets, moles, and field mice; sheep, goats, swine, rabbits, white mice, and house mice are much less susceptible; cattle are immune. Man is susceptible, developing both the acute and chronic forms. Infection not infrequently terminates fatally, usually in about 60 per cent. of the cases. Doubtless many cases are not recognized as glanders, but are mistaken for other diseases such as pyemia, rheumatism, typhoid and syphilis. (Fitch.)

When pure cultures of *Bacillus mallei* are injected into horses or other susceptible animals true glanders is produced. The disease is characterized in the horse by the formation of ulcers upon the nasal mucous membrane, which have irregular, thickened margins, and secrete a thin, virulent mucus; the submaxillary lymphatic glands become enlarged and form tumors which are often lobulated; other lymphatic glands become inflamed, and some of them suppurate and open externally, leaving deep, open ulcers; the lungs are also involved, and the breathing becomes rapid and irregular. Acute generalized glanders may cause death in one to six weeks, young horses being

especially susceptible. At postmortem usually the lungs show either tubercle-like nodules or pneumonic areas. Nodules may occur also in the liver, spleen and lymph glands. Of the bones, the ribs are the most often involved and contain caverns filled with a tenacious yellowish substance. In *farcy*, which is a more chronic form of the disease, circumscribed swellings, varying in size from a pea to a hazel-nut, appear on different parts of the body, especially where the skin is thinnest; these suppurate and leave angry-looking ulcers with ragged edges, from which there is an abundant purulent discharge. The lymphatics leading from these ulcers become inflamed, stand out as tense hot cords under the skin and from them new "farcy buds" may develop. Chronic cases may run on for years until an acute exacerbation due to overwork or adverse conditions brings death.

The bacillus of glanders can be obtained in pure cultures from the interior of suppurating nodules and glands which have not yet opened to the surface, and the same material may give successful results when inoculated into susceptible animals. The discharge from the nostrils or from an open ulcer may contain comparatively few bacilli, and these being associated with other bacteria which grow more readily on the culture media than the *Bacillus mallei*, make it difficult to obtain pure cultures from such material by the plate method. In that case, however, guinea-pig inoculations are useful.

Of test animals guinea-pigs and field mice are the most susceptible. In guinea-pigs subcutaneous injections are followed in four or five days by swelling at the point of inoculation, and a tumor with caseous contents soon develops; then ulceration of the skin takes place and a chronic purulent ulcer is formed. The essential lesion is the granulomatous tumor, characterized by the presence of numerous lymphoid and epithelioid cells, among and in which are seen the glanders bacilli. The lymphatic glands become inflamed and general symptoms of infection are developed in from two to four weeks; the glands suppurate, and in males the testicles are involved. This fact is used as a means of diagnosis. (See Straus Reaction, p. 406.) Finally purulent inflammation of the joints occurs and death ensues from exhaustion. The formation of the specific ulcers upon the nasal mucous membrane, which characterizes the disease in the horse, is rarely seen when guinea-pigs are inoculated. In these the process of the disease is often prolonged or remains localized on the skin. Guinea-pigs succumb more rapidly to intraperitoneal injection, usually in from eight to ten days.

Attenuation of virulence occurs in cultures which have been kept for some time on artificial media and inoculation with such cultures may give a negative result, or, when considerable quantities are injected, may produce a fatal result at a later date than is usual when small amounts of a recently isolated culture are injected.

**Mode of Spread.**—Glanders occurs as a natural infection only in horses and asses. The disease is occasionally communicated to man by contact with affected animals, usually by inoculation on an abraded surface of the skin. The contagion may also be received on the mucous

membrane. Infection has sometimes been produced in bacteriological laboratories. It is transmissible also from man to man. Washerwomen have been infected from the clothes of a patient. The infective material exists in the secretions of the nose, in the pus of glanders nodules, and frequently in the blood; it may occasionally be found in the secretions of glands not yet affected, as in the urine, milk, and saliva, and also in the fetus of diseased animals (Bonome). From recent observations it is found that glanders is by no means an uncommon disease among apparently sound horses, sometimes taking a mild course and remaining latent for a considerable time. Therefore, horses appearing healthy, may spread the disease through the public drinking troughs and blacksmith shops.

**Immunity.**—Attempts have been made to produce artificial immunity against glanders but so far with unsatisfactory results. Various workers, as Straus, Fenger and also Ladowski, have reported the production of immunity in the smaller animals, such as dogs, cats and rabbits, by the injection of either living or killed cultures. In the horse, the most important animal from the economic standpoint, other observers have reported not only immunity but cures by the use of vaccine and also subcutaneous mallein; however, corroboration is still lacking. Such substances as mallein and vaccine when injected do produce immune bodies which can be demonstrated by the serodiagnostic tests. It is unfortunate, however, that the presence of these immune bodies in the blood does not indicate, necessarily, a practical immunity of the animal against infection. (Mohler and Eichhorn.)

Since the indiscriminate use of *vaccine* causes confusion in the blood tests of horses, it is not now being distributed by the Health Department of the City of New York.

In man the therapeutic value of vaccine is not yet fully determined. But few cases so far have been reported—one by Bristow and White and one by Cramp recovered after the use of an autogenous glanders vaccine. The use of *mallein* in man subcutaneously, has been reported in six cases. (Robins.) In one it gave a reaction and in three it was of supposed benefit therapeutically.

**Diagnosis of Glanders.**—The chief methods (Mohler and Eichhorn) are: physical examination; serodiagnostic tests—complement-fixation and agglutination reaction; mallein reactions—eye and subcutaneous; “Straus reaction” (inoculation of guinea-pigs with either suspected material or cultures); postmortem examination.

**Physical Examination.**—In horses those cases with clear-cut clinical symptoms (p. 400) offer little difficulty to the veterinarian. However, the easily applied eye mallein reaction should be used for confirmation. These two methods are sufficient under such circumstances. It is the latent or occult cases, showing only a little fever, or none, that require additional tests for diagnosis. Since these cases are frequently the distributors of the diseases their early recognition and extermination is imperative. To detect these cases the application of the serodiagnostic reactions supplemented by the use of eye mallein is necessary (p. 404).

**Collection of Blood for Serodiagnostic Tests.**—In obtaining blood from horses a large-sized hypodermic needle, which has been sterilized, is inserted into the jugular vein which has been brought into view by pressing the thumb upon it from below; the blood is allowed to flow through the needle into a sterile neutralized tube or flask, 8 to 10 c.c. being sufficient.

In the case of human beings, the median basilic vein at the bend of the elbow is used. Under aseptic conditions 5 to 10 c.c. of blood are drawn either by means of a sterile hypodermic syringe, or allowed to flow through a large hypodermic needle, as above.

**Complement-fixation Test.**—In 1909 Schütz and Schubert applied this method to the diagnosis of glanders. It gives excellent results, for it picks up 97 per cent. of positive cases according to Miessner and Trapp. Its failures lie chiefly in the early stages of the disease (see p. 406), and for this reason it should be paralleled by the agglutination reaction. The combination of these two tests gives, according to Huytera and Marek, a percentage of 99 successful tests. The use of a polyvalent antigen for the detection of glanders, as of gonorrhea, is important.

**Agglutination Reaction.**—It was first applied by MacFadyean (1896) who used the microscopic method as in the Widal reaction. Later Schütz and Miessner (1896) found the macroscopic method more practicable. In the early stages of glanders this method is most valuable (see p. 406). It picks up about 84 per cent. of positive cases (Anthony and Grund), the failures occurring chiefly in old chronic cases.

Schütz and Miessner claim that a culture recently passed through a guinea-pig (once in three weeks) is essential for a good test fluid. In our hands cultures kept on artificial media do well if passed through a pig once in two months. Not every strain of *B. mallei* agglutinates well, consequently a suitable one must be chosen. A univalent test fluid is the most logical, for in a polyvalent fluid a strain not agglutinated by a particular serum would cloud the supernatant fluid at the same time that a reaction occurred in the bottom of the tube.

The *macroscopic agglutination* test may be carried out in several ways:

**Incubator Method.**—The procedure of Schütz and Miessner, with slight modifications, is as follows: A forty-eight-hour acid-glycerin-agar culture of *B. mallei* is washed off with normal saline solution containing 0.5 per cent. pure carbolic acid. This suspension is heated at 60° C. for two hours. It is then filtered through cotton, and enough of the carbolic salt solution added to reduce it to a faintly cloudy suspension. This should be standardized by comparing it with a known test fluid, if possible, and testing it with known negative and positive sera. This test fluid will keep in the ice-box for several weeks.

The active serum to be tested is then made up with normal salt solution to a 1 to 40 dilution. From this the final dilutions of 1 to 500, 1 to 800, 1 to 1000, etc., are made by adding 0.24 c.c., 0.15 c.c., and 0.12 c.c. respectively to 3 c.c. of the standardized test fluid in each test-tube. The tubes are well shaken and incubated twenty-four to seventy-two hours with positive and negative control sera in the same dilutions. If a reaction occurs the upper part of the fluid is clear, while a veil-like sediment is found at the bottom. A strong positive reaction (1 to 1000) may occur in twenty-four hours. A negative reaction shows the sediment in a definite "button" at the bottom of the tube and the fluid above is cloudy.

With the *centrifuge method* of Miessner and others, cited by Mohler and Eichhorn the time factor is greatly reduced. The tests are incubated for a half-hour at 37° C., then centrifugalized at 1600 revolutions for ten minutes, and kept at room temperature for two hours before reading. This is done by looking down on the tubes from above toward a dark background. Indefinite reactions may be read the next day after standing at room temperature. Special tubes with perfectly rounded bottoms are essential for this method, and the dilutions are made up in only 2 c.c. of the test fluid.

**Rapid Method.**—Povitzky, of this laboratory, is applying the following method of macroscopic agglutination with time-saving results. Technic: A fresh culture grown for forty-eight hours on glycerin-potato-veal agar (2.5 acid to phenolphthalein) is washed off with a small amount of sterile normal salt solution. This thick milky suspension is heated for one hour at 70° C. After filtering through cotton, normal salt solution is added until the suspension is only faintly cloudy. This test fluid must be standardized by comparison with a known standard, etc., as described under the *incubator method* of agglutination. The active serum to be tested is then diluted with normal salt solution and added in suitable amounts to 3 c.c. of the test fluid (see procedure under incubator method). The various dilutions and controls are then placed in a water-bath at 37° to 40° C. for two hours. Very active reactions can be read as early as the end of the first hour; others at the end of the second hour. The tubes are then set in the ice-box overnight and any delayed reactions may be read next morning. Although this method is only a little more rapid in time than the centrifuge method, its easier technic and lack of complicated apparatus recommend it.

The limit of agglutination in the normal horse is 1 to 500, most of the reactions occurring at 1 to 200 or 400. Since, however, some cases of chronic glanders do not react above 1 to 500, this reaction should be regarded with suspicion and checked by the complement-fixation test and eye mallein; so also reactions below 1 to 1000. Reactions of 1 to 1000 are positive, some horses running up to 1 to 2000 or 3000. In practical work dilutions higher than 1 to 1000 are unnecessary and this test should always be checked by the use of eye mallein and the complement-fixation test.

In human cases a reaction by the "rapid method" (Povitzky) of 1 to 500 and above, is considered positive. Normal human blood reacts seldom above 1 to 100, but it may reach 1 to 200 or even 400 in exceptional cases.

**Mallein Reaction.**—Mallein is like tuberculin in that it consists of glycerinated bouillon which contains the products of the growth and activity of *B. mallei* cultivated in it. It was discovered by Kelnig, a Russian veterinarian, in 1890. (For the preparation of the two kinds of mallein—*eye* and *subcutaneous*, see p. 406.) The *eye mallein reaction* is the most recently developed test for glanders in animals, yet it has taken a preëminent place in diagnosis for it is already the Federal test for the interstate shipment of horses. The simplicity of the application of eye mallein, the short time—twenty-four hours—required, and the comparatively easy reading of results, after a suitable experience, make it possible for the veterinarian to apply prompt tests on suspected horses.

In healthy horses the error of this test has been shown (Schnarer) to be only 0.39 per cent., while in glanderous horses the test gives 88.8 per cent. positive, 3.5 per cent. negative, and 7.5 per cent. doubtful.

As with the complement-fixation test the doubtful and negative reactions occur chiefly in the early stages of the disease; consequently its use alone, without the agglutination reaction, to check the complement-fixation test is subject to error unless retests at suitable intervals are planned and carried out.

*Technic of Application.*—When 2 or 3 drops of concentrated mallein are instilled into the conjunctival sac, no reaction save a slight lacrimation and congestion results in healthy horses. In glanderous animals this goes on, at the end of from five to seven hours, to profuse lacrimation, redness, edema, and the formation of pus. There may be only a drop of pus at the inner canthus of the eye, or all degrees to profuse purulent discharge. Unless pus is present, the reaction is not considered positive. There is a slight rise in temperature in those cases showing a marked reaction, but as the local reaction is very distinct, the tedious task of taking temperatures as in the subcutaneous method is superfluous.

Another advantage of this method over the subcutaneous inoculation, aside from its simplicity, is the fact that it can be repeated after twenty-four hours in doubtful cases; also it does not interfere with subsequent serodiagnostic tests. With very few exceptions, a second test in a glanderous horse gives a prompt reaction.

*Subcutaneous Infection of Mallein.*—Although the injection of mallein subcutaneously is one of the oldest and most reliable methods for the diagnosis of glanders it should be applied only after the complement-fixation, agglutination and eye mallein tests have been used, since the subcutaneous injection of mallein, as also any glanders antigen including vaccines, interferes with the serodiagnostic tests. In spite of the fact that it picks up 89 per cent. (Huytera and Marek) of the positive cases, its cumbersome technic of prolonged temperature taking, the detention of the horses from work, etc., all contribute to render it unsuitable as an early test.

Before applying the subcutaneous test the temperature of the horse should be taken at least three times at intervals of three hours. If there is fever the mallein should not be given. The injection of mallein (usually about 2 c.c.) should be made about 10 P.M. In a glanderous horse there will be a local reaction and a general reaction with fever. The temperature begins to rise usually three or four hours after the injection and reaches its maximum between the tenth and twelfth hours. Sometimes the highest point is not reached until fifteen to eighteen hours after the injection. This rise in temperature is from 1.5° to 2° C. (2° to 3.5° F.). The temperature taking should be continued every two hours, beginning not later than eight to ten hours after the mallein was given. The general condition of the animal is more or less profoundly modified and the local reaction is usually very marked around the point of injection. Here, in a few hours, there appears a warm, tense and very painful swelling. Running from this will be found hot, sensitive lines of sinuous lymphatics directed toward the neighboring lymphatic nodes. This edema increases for twenty-four to thirty-six hours and persists for several days, not disappearing entirely for eight or ten days.

In healthy animals the rise of temperature is usually only a few tenths of a degree but it may reach 1° C. This rise should always be considered, however, in connection with the general and local reactions. At the point of injection the mallein produces only a small edematous tumor which, instead of increasing diminishes rapidly and disappears in about twenty-four hours.



**Occurrence of the Reactions of the Various Tests after Infection.**—*Agglutinins* increase above normal in four or five days and continue to rise in the early stages of the disease, diminishing as the disease becomes chronic. Specific amboceptors for the *complement-fixation* test may be demonstrated in from seven to ten days and remain during the entire course of the disease. The *subcutaneous mallein* test may, as a rule, be relied upon for diagnosis fifteen days after infection, while the *eye mallein test* is reliable twenty-one days after infection. (See Report on Detection on Glanders, in References at end of this chapter.)

**Effect of One Test on the Others.**—The serodiagnostic tests are influenced in three to six days after a subcutaneous injection of mallein or any glanders antigen including vaccines. The period of influence varies from six to eight weeks after the injection of mallein, and lasts three months or longer after the injection of glanders antigen or vaccines.

**"Straus Reaction."**—This test consists of introducing into the peritoneal cavity of a male guinea-pig some material or a culture from the suspected products. If the *B. mallei* is present, the diagnosis may usually be made within two to five days from the tumefaction of the testicles showing evidences of pus formation from which pure cultures can be obtained. An objection to this method, however, is that occasionally from the injection of impure material, as in the nasal secretion, the animal may die of septicemia; but if an uncontaminated specimen can be obtained, as from the lymphatic glands, this method is satisfactory according to most authors. Nevertheless, while a positive result is conclusive evidence of glanders, failure of the pig to develop lesions is not proof of its absence, for other workers find such inoculations fail in about one-half the animals injected, even after a prolonged period.

**Postmortem Lesions.**—Postmortem lesions are given on page 400. The confirmation of the findings of all positive (or doubtful) tests by careful examinations at autopsy is most desirable in order to extend our present data along these lines.

**Preparation of Mallein.**<sup>1</sup>—Mallein for *subcutaneous injection* is produced by growing *B. mallei* (preferably a variety of strains) for six to eight weeks in a 5 per cent. glycerin-nutrient veal bouillon, about 2.5 acid to phenolphthalein. (See chapter on Media, p. 110.) Each flask or bottle of the culture is then tested for purity by the examination of smears and cultures made on *neutral* veal agar—a medium unfavorable to the growth of *B. mallei*. If pure, the broth culture is killed by steaming in the Arnold sterilizer for one hour. After sedimentation in the ice-box for a few days the supernatant fluid is filtered first through paper pulp and then through the Berkefeld filter. Carbolic acid is added to give 0.5 per cent.

For *eye mallein* the same procedure as above is followed except that after filtering the liquid through paper pulp, it is measured and then evaporated over the hot-water bath to one-tenth its volume. The viscid liquid is then sterilized by heating in the Arnold sterilizer for three-

<sup>1</sup> The methods given are those in use in the Health Department of the City of New York.

quarters of an hour. The precipitate which has formed is thrown down either by centrifugalizing, or by sedimentation in the ice-box. This latter method takes about two weeks.

Before use both the *subcutaneous* and the *eye* mallein should be subjected to potency tests on both glandered and normal horses.

### B. ABORTUS (BANG).

This organism, first described by Bang, is the cause of contagious abortion in cattle. It is a small, pleomorphic Gram-negative bacillus which when first isolated is micro-aërophilic, becoming aërobic on cultivation. Its main interest medically is its common presence in milk and the possibility of human infection arising from this source. The presence in the blood of agglutinins and complement-fixing substances has been demonstrated in an appreciable number of children and in some instances of aborting women (Larson and Sedgwick, Nicoll and Pratt). Only once, however, has the organism been isolated from human tissues, viz., a tonsil, which cannot be considered as an infection. Whether such antibody reactions are due to intestinal absorption of the products of the bacilli contained in the milk or to the passive absorption of antibodies (Cooledge) in the milk of infected cows cannot be answered. At least it does not seem that they are due to infection.

The other interest is that *B. abortus* when injected into guinea-pigs gives rise to lesions very similar to those of tuberculous origin (Smith and Fabyan). It is evident that this may be a source of error in examining milk for the presence of tubercle bacilli by inoculation. Spontaneous infection of guinea-pigs has also occurred in a laboratory, the source of infection being inoculated pigs.

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## CHAPTER XXX.

### THE GROUP OF HEMOGLOBINOPHILIC BACILLI. BORDET-GENGOU BACILLUS.

#### THE INFLUENZA BACILLUS.

A DISEASE called influenza can be traced back to the fifteenth century and probably existed at a much earlier date.

At times but few endemic cases occur, and then a great epidemic spreads over the civilized world. The last great epidemic reached Russia from the East in the fall of 1889 and gradually spread over Europe and to America, reaching the latter country in December of that year. Since then we have had more or less of it, especially during the winter months. Many acute inflammations of the respiratory mucous membranes due to pneumococci and streptococci, give symptoms similar to those due to the influenza bacillus.

The rapidity of the spread of epidemics of influenza suggested that persons were the carriers of the infection, while the location of the disease pointed to the respiratory tract as the location of, and to the expectoration as the chief source of infection by, the microorganisms.

After numerous unsuccessful attempts, during the epidemic of 1889 and succeeding years, to discover the specific cause of influenza, Pfeiffer (1892) succeeded in isolating and growing upon blood agar a bacillus which abounded in

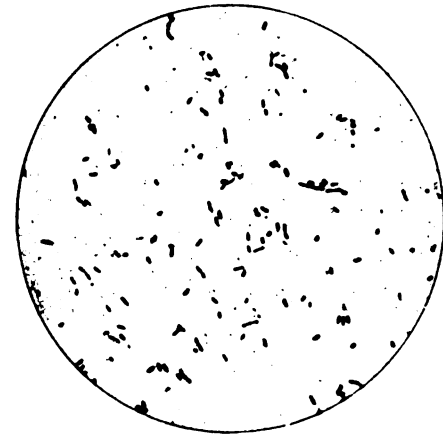


FIG. 143.—Influenza bacilli.  $\times 1100$  diameters.

the purulent bronchial secretion of patients suffering from epidemic influenza, which he showed was the probable cause of the disease. Canon, working at the same time, found a similar bacillus in the blood of several cases of the disease. Though *B. influenzae* has been shown to have definite pathogenic powers, its specificity in epidemic influenza has not been fully proved.

**Morphology.**—Very small, moderately thick bacilli ( $0.2\mu$  to  $0.3\mu$ ) in thickness to  $0.5\mu$  to  $2\mu$  in length), usually occurring singly or united in pairs, and occasionally showing threads, are found in spreads from the

sputum and young cultures. In later cultures threads may be produced in great abundance. No capsule has been demonstrated.

**Staining.**—The bacillus *stains* rather faintly with the ordinary aniline colors—best with dilute Ziehl's solution (water 9 parts to Ziehl's solution 1 part), or Löffler's methylene-blue solution, with heat. Giemsa's method stains them well and brings out the polar granules which sometimes develop in these bacilli. They are not stained by Gram's method.

**Biology.**—An aërobic, facultative anaërobic (contrary to the accepted opinion), non-motile bacillus; does not form spores; no growth occurs with most cultures below 22° C., or above 41° C.

**Cultivation.**—This bacillus is best cultivated at 37° C., and on ordinary nutrient culture media containing hemoglobin (p. 103). At the end of eighteen hours in the incubator very small circular colonies are developed, which, under a low magnification (100 diameters), appear as shining, transparent, homogeneous masses, and even at 600 diameters scarcely show indications of the individual organisms. Older colonies are sometimes colored yellowish brown and in the centre are characteristic heapings. Another characteristic feature of the influenza bacillus is that the colonies tend to remain separate from each other, although when they are thickly sown in a film of moist blood upon nutrient agar they may become confluent. Transplantation of the original culture to ordinary agar or serum cannot be successfully performed, owing to the want of sufficient hemoglobin; but if sterile rabbit, pigeon, horse or human blood be added to these media, even in very small amounts (1 to 500), especially if the agar is very hot (90° C.) at the time the blood is added, transplantation may be indefinitely performed, provided it is done every three or four days. Cultures if kept moist, may remain alive for a variable time. By a series of beautifully carried out experiments Pfeiffer showed that not only were the red blood cells the necessary part of the blood needed for the growth of the influenza bacillus, but that it was the hemoglobin in the cells that was the essential element.

Though in pure cultures this organism will not grow without hemoglobin, in the presence of certain other organisms it will grow abundantly for successive culture generations without blood. Meissner and others found that it would grow well with members of the diphtheria group, and with the pyogenic cocci. We have found, contrary to others, that staphylococci tend to inhibit growth.

**Resistance and Length of Life.**—The influenza bacillus is very sensitive to desiccation; a pure culture diluted with water and dried is destroyed with certainty in twenty-four hours; in dried sputum the vitality, according to the completeness of drying, is retained from twelve to forty-eight hours. It does not grow, and soon dies in water. In blood-bouillon cultures at 20° C. it retains its vitality for several weeks. In moist sputum it is difficult to determine the duration of its life, since the other bacteria overgrow and make it impossible to find it. It probably can remain alive for at least two weeks. The bacilli are

very readily killed by chemicals, disinfectants, and succumb to boiling within one minute and to 60° C. within five minutes.

**Effect on Animals.**—The bacillus of influenza is only slightly virulent for experimental animals. It is, however, definitely though moderately pathogenic for some animals, especially the rabbit, and several observers have found that such pathogenic power (from one blood-agar slant to  $\frac{1}{10}$  of a slant) may be decidedly increased by successive passage through the susceptible animal. Guinea-pigs are also quite susceptible to some strains. Pure, abundant cultures are always obtained from the heart after intraperitoneal inoculation. Wollstein has been able to produce cerebrospinal meningitis in the monkey by subdural inoculation. Toxic products cause toxic symptoms in rabbits only when inoculated in large quantities.

**Immunity.**—Short immunity may be established after an attack, though in some cases animals seem to be more sensitive to a second attack. That rabbits may be hyperimmunized is shown in the process of obtaining antibodies. At least in three experiments made by Pfeiffer on monkeys, these animals, after recovering from an inoculation with bacilli, seemed to be much less susceptible to a second injection.

**Complement-fixation.**—Specific antibodies may be obtained in most rabbits, which definitely fix complement. The greatest difficulty in demonstrating the phenomenon of complement-fixation with this group of organisms, is the preparation of suitable antigens. We have found that with the use of only small quantities of heated blood in the medium and by employing the method of shaking the cultures and then incubating overnight to help the autolysis, very satisfactory antigens have been obtained. Definite groups among these bacilli have been demonstrated by this reaction.

**Pathogenicity for Man.**—The invasion of the body by the influenza bacillus is more widespread than was earlier supposed. Very frequently the influenzal process invades portions of the lung tissue. In severe cases a form of pneumonia is the result, which is lobular and purulent in character and accompanied by symptoms which may be somewhat characteristic for influenza, or, again, almost identical with bronchopneumonia due to the pneumococcus. The walls of the bronchioles and alveolar septa become densely infiltrated with leukocytes, and the spaces of the bronchial tubes and alveoli become filled. The influenza bacilli are found crowded in between the epithelial and pus cells and also penetrate the latter. There may be partial softening of the tissues or even abscess formation. Bacilli are found in fatal cases to have penetrated from the bronchial tubes not only into the peribronchitic tissue, but even to the surface of the pleura, and in a few cases they have been obtained in pure cultures in the pleuritic exudation. The pleurisy which follows influenza, however, is usually a secondary infection, due to the streptococcus or pneumococcus.

**Presence in Other Parts of the Body.**—Influenza bacilli are found at times in the blood during the early days of an acute infection, while there is marked fever (Ghedini found them in 50 per cent. of his cases);

and sometimes in bad cases in young children a septicemia develops before death. It is found at times in otitis media accompanying influenza, and has been found in cases of meningitis (Wollstein), in many cases of acute and subacute conjunctivitis (Williams) and in several cases of peritonitis, appendicitis, and cystitis. The general, cerebral, gastric, and other symptoms produced are usually due to the absorption of the toxic products of the specific organism, these poisons being particularly active in their effects on the central nervous system.

**Presence of Influenza Bacilli in Chronic Influenza and in Tuberculosis.**—Ordinarily influenza runs an acute or subacute course, and not infrequently it is accompanied by mixed infections with the pneumococcus and streptococcus. Pfeiffer was the first to draw attention to certain chronic conditions depending upon the influenza bacillus. Bacilli may be retained in the lung tissue for months at a time, remaining latent a while, and then becoming active again, with a resulting exacerbation of the disease. Consumptives frequently carry influenza bacilli for years and are particularly susceptible to attacks of influenza. Williams, in the examination of sputa in cases of pulmonary tuberculosis, found abundant influenza bacilli to be present in a large proportion of the samples of sputum from consumptives, and this not only in winter but also in the summer, when no influenza was known to be present in New York. Taken together with results elsewhere, this indicates that at all times of the year many consumptives carry about with them influenza bacilli, and that very likely many healthy persons as well as persons suffering from bronchitis also harbor a few. Given favorable conditions, we have at all times the seed to start an epidemic.

**Epidemiology.**—The discovery of this bacillus enables us to explain many things, previously unaccountable, in the cause of epidemic influenza. We now know, from the inability of the influenza bacillus to exist for long periods in dust, that the disease is not transmissible for great distances through the air. We also know that the infective material is contained only in the secretions. Sporadic cases or the sudden eruption of epidemics in any localities from which the disease has been absent for a long time, or where there has been no new importation of infection, may possibly be explained by assuming that the bacilli, as already mentioned, often remain latent in the lungs or bronchial secretions or secretions of the conjunctiva for many months, and perhaps years, and then become active again, when under favorable circumstances they may be communicated to others.

**Bacteriological Diagnosis.**—This is of importance for the identification of clinically doubtful cases, which, from their symptoms, may be mistaken for bronchitis, pneumonia, or tuberculosis.

In acute uncomplicated cases the probable diagnosis can be frequently made by microscopic examinations of stained preparations of the sputum. In chronic cases or those of mixed infection few or many bacilli may be found and the culture method may be necessary to give even a probable diagnosis. The bacillus of influenza is not readily separated by its morphological, staining, and cultural peculiarities from

other bacteria belonging to the influenza group, and at present by the spread method it is almost impossible to identify it certainly.

**Examination of Sputum for Influenza Bacilli.**—1. Sputum coughed from the deeper air passages and not from throat scraping should be used.

2. The sputum should be expectorated into a sterile bottle, which should then be placed immediately in cracked ice to transport to the laboratory.

3. Blood-agar plates should be made by placing a drop of fresh rabbit or horse blood, obtained aseptically, on the centre of a hardened agar plate.

4. One of the more solid masses of the sputum should be taken from the bottle with sterile forceps and placed on a plain agar plate. A small portion of this mass should be separated with a sterile platinum needle and drawn through the blood on the blood-agar plate from the centre out in different directions. The larger part of what is left of this small portion is then placed in a similar manner over a second blood agar, and from this to a third, sterilizing the needle between the transfers. The plates should be placed in the incubator at about 36° C. for twenty-four hours.

5. After the plates are planted two smears should be made, from the sputum, one stained by Gram and the other by weak carbol-fuchsin.

6. After twenty-four hours the plates are examined under low power. The influenza colonies use up the hemoglobin, and in parts of the blood-agar plate where the blood is of right thickness such colonies show as almost clear white areas surrounded by the red blood. With a higher power (No. 6 or 7 objective), if such areas seem to be made up of fine indefinite granulations, they are practically sure to be influenza colonies. Most influenza colonies are more highly refractive than other light colonies, and they show this characteristic best when they grow on the edge of a blood mass. Many influenza colonies also show heapings in the centre. Influenza colonies growing away from the blood cells are less characteristic in appearance and less easily differentiated from other similar bacteria.

7. Fishings from the influenza-like colonies should be planted on blood-agar tubes, and if, after twenty-four hours in the thermostat, the resulting growth should consist of influenza-like organisms, plantings should be made on plain agar. The first generation on plain agar may show slight growth because of the blood carried over from the original tube, but the second generation should show no growth if the organism is the influenza bacillus.

8. The agglutination characteristics of the cultures should be tested in the serum from a rabbit injected with a single typical culture, and in the serum from one injected with a number of cultures. The agglutination tests should be carried out in order to gain knowledge in regard to their worth. The cultures tested in the Research Laboratory have shown considerable variation.

**Other Bacilli Resembling the Influenza Bacillus.**—There are a number of bacilli which differ slightly in morphology and growth in culture from the characteristics of the typical influenza bacillus. These were grouped under the name "*pseudo-influenza bacilli*." But so far there have been shown no characteristics distinct enough to separate these hemoglobinophilic organisms into other than "strains" or "varieties" of the one specie, *B. influenzae*. For example, the influenza-like bacilli found first in whooping-cough by Jochmann and others, Müller's "trachoma bacillus," Koch-Weeks' bacilli, the bacilli found by Cohen in meningitis, and those reported occasionally in other parts of the body—all of them seem to be so closely related that they should be considered one species or, at the most, varieties of one species until more specific characteristics can be demonstrated.

**Relation of the Clinical Symptoms to the Bacterial Excitant.**—There is no doubt that other infections are also included under the clinical forms of influenza, and during an epidemic of bronchopneumonia, irregular types of lobar pneumonia, and cases of bronchitis frequently have symptoms so closely alike that the nature of the bacteria active in the case is very frequently different from that supposed by the clinician. Thus in four consecutive autopsies examined by the writers the influenza bacillus was found almost in pure culture in one case believed, from the symptoms, to be due to the pneumococcus, and entirely absent in two of the three believed to be due to it. Except for these examinations the clinician would be of the opinion that he had clearly diagnosed bacteriologically the cases, while in fact he had been wrong in three of the four.

The striking symptoms in acute respiratory diseases are frequently due more to the location of the lesions than to the special variety of organisms producing them. In epidemics of influenza there are, of course, many cases which, on account of their characteristic symptoms, can be fairly certainly attributed to the influenza bacillus. Even under these circumstances error may be made, as, for instance, two cases of apparently typical influenza were reported in a household and both showed a total absence of influenza bacilli. The pneumococcus was present in almost pure culture.

**For Testing the Agglutination of Influenza Bacilli in the Hanging Drop.**—Grow the cultures on coagulated blood-agar slants (see p. 409). When twenty to twenty-four hours old, make a suspension of the bacilli in normal salt solution, controlling the number of bacilli by examining a hanging-drop preparation. The influenza bacilli agglutinate rather slowly, so it usually takes four or five hours to get a good reaction.

**Serum Therapeutics.**—No protective serum has been produced which has been used successfully in human cases, but Wollstein obtained one which was of value in experimentally produced infection in monkeys.

**Vaccine Treatment.**—So far this has not been proven to be of marked value. Certain cases may be slightly helped, but too little is still known about the best conditions for use to make it of universal value. Sensitized vaccines have been said to give good results.

### INFLUENZA-LIKE BACILLI IN CONJUNCTIVITIS (INCLUDING TRACHOMA).

**The Koch-Weeks' Bacillus.**—This bacillus was first observed by R. Koch in 1883 while making certain investigations into inflammation of the eye occurring during an epidemic of cholera in Alexandria. It was later, in 1887, more specifically described by Weeks in New York. Weeks obtained it in cultures growing with the xerosis bacillus from cases of "pink-eye," or acute contagious conjunctivitis. Morax stated that he was able to obtain pure cultures only until the third culture



generation. Others state that on human serum or hydrocele fluid they have obtained growths for many generations. Kamen concludes that it is a strict hemoglobinophile. Our studies led us to agree with this last conclusion. The successive cultures obtained with some sera are probably due to the presence of hemoglobin in amounts too small to be easily detected, but large enough to allow growths of hemoglobinophilic bacilli. The few differential points claimed between this bacillus and influenza bacilli do not hold (Williams) and so the question of their relationship is not settled.



FIG. 144.—Koch-Weeks' bacillus from ("pink-eye")—third generation.  $\times 1000$  diameters. (Weeks.)



FIG. 145.—Secretion of mucopus from conjunctiva in "pink-eye."  $\times 1000$  diameters. (Weeks.)

**Other Microorganisms in Conjunctivitis.**—Many organisms are found in diseases of the eye, but few of these present evidence of specific etiology.

**Morax-Axenfeld Bacillus.**—In certain subacute inflammations of the conjunctiva, especially noticeable about the angle of the eyes (angular conjunctivitis), Morax (1896) and later Axenfeld found a bacillus which they consider the cause of the disease.

**Morphology.**—Short (about  $2\mu$  long), thick, non-motile bacilli, generally in twos, but sometimes single or in short chains. They take the ordinary stains easily, but are decolorized by Gram's stain.

**Cultures.**—At  $37^{\circ}\text{C}$ . the bacilli produce a delicate growth on media containing blood or serum. Later cultures grow slightly on nutrient veal agar. They grow slightly, if at all, at room temperature.

Upon serum agar, they form delicate grayish colonies.

Upon Löffler's blood serum after twenty-four to thirty-six hours the growth appears as an indentation of the medium due to liquefaction. This liquefaction continues slowly for a variable time.

In ascitic broth cloudiness is produced within twenty-four hours.

**Pathogenicity.**—Lower animals so far have shown themselves refractory. In human beings inoculations of pure cultures have produced subacute conjunctivitis.

**Bacillus of Zur Nedden.**—This bacillus has been found by Zur Nedden in certain ulcers of the cornea and is supposed to be the etiological factor in this disease.

**Morphology.**—Small (usually less than  $1\mu$  long) rather slender single, sometimes slightly curved, non-motile bacilli. They may occur as diplobacilli, but they do not form chains.

They stain easily, sometimes faintly at ends. They are decolorized by Gram.

**Cultures.**—They are easily grown on all laboratory media. Upon agar, within twenty-four hours they produce rounded, raised, translucent, slightly fluorescent colonies, which are more or less confluent and, under the low power, are rather coarsely granular. Upon *potato* they form a thick, yellowish growth. *Milk* is coagulated; *gelatin* is not liquefied; dextrose is fermented without gas. No indol is produced.

**Pathogenicity.**—Pure cultures inoculated in the cornea of guinea-pigs produced ulcers.

**Trachoma.**—Many studies have been made on the etiology of trachoma (progressive follicular inflammation of the conjunctiva followed by cicatrization) and allied conjunctival affections. Halberstädter and Prowazek (1907) state that the cause of trachoma is a small germ which grows in a characteristic way in the conjunctival epithelial cells. The organism itself, they say, is so small that at first it cannot be seen, only the mantle which it produces is demonstrable. This stains blue with Giemsa, and as the organisms grow in bunches, one sees at first in the neighborhood of the nucleus only a bunch of small, blue, coccus-like bodies. The organism finally appears as a minute red granule within the blue body. As it continues to increase in numbers and size, the blue mantles finally disappear, leaving a mass of small rounded or slightly elongated red bodies. The bodies are only found in the early acute cases. Prowazek named them *Chlamydozoa* on account of their mantle, and thinks they should occupy a place between bacteria and protozoa.

Our studies given below show that there is similarity of these inclusions to nests of growing hemoglobinophilic bacilli.

The Koch-Weeks' bacillus has been frequently reported as occurring in trachomatous eyes, from the time of Koch (Collins, Morax, Müller, etc.). Markus states that the Koch-Weeks' bacillus is the cause of "Schwellungskatarrh" (our papillary conjunctivitis). Mütermilch goes further in declaring that "often repeated infection with the same microorganism, *e. g.*, bacillus of Koch-Weeks, produces a series of exacerbations on an already inflamed conjunctiva and finally produces the picture of trachoma."

Müller, who isolated hemoglobinophilic bacilli from the largest series of trachoma cases reported, had positive results chiefly in his "acute trachoma" cases. Müller thought at first that his bacillus was the cause of trachoma, but others thought that it was an influenza bacillus and had nothing specifically to do with trachoma (Zur Nedden, Morax and others).

We, however, agree partly with both workers. We have demonstrated the continued presence of hemoglobinophilic bacilli in cases showing successively acute, subacute, and chronic inflammation of the conjunctiva and their increase in numbers during acute exacerbations of chronic cases. We have also shown that the bacilli found in cases of "pink-eye" are indistinguishable, thus far, from hemoglobinophilic bacilli found in the chronic cases. Furthermore, we have pointed out a relationship between hemoglobinophilic bacilli and trachoma inclusions.

In studying closely the morphology in cultures of these bacilli, we were struck by the fact that they frequently grew in more or less dense clumps of extremely minute and irregular coccoid forms. This led us to the conclusion that possibly they form the Prowazek inclusions found in trachoma, and when we found that these bacilli and the inclusions were found coincidentally and repeatedly in so many cases diagnosed as acute papillary trachoma (called by us papillary conjunctivitis), the possibility became a probability and we proceeded to study the morphology of the cultures more minutely.

The morphology varies somewhat with the age of the inoculated culture, the number of culture generations, the kind of medium, and the strain. After forty-eight hours' incubation the forms become somewhat more irregular. Then in three days most of the bacilli have become extremely minute, many showing only as reddish granules (the "elementary bodies of Prowazek"), while scattered through the culture are swollen spheroidal bodies taking a fainter clear blue stain (the larger "initial bodies of Prowazek"), in some of which are minute reddish granules (more of the "elementary bodies of Prowazek"). A number of irregular light blue bodies are also scattered through the culture. Where the bacteria are densely grouped more red granules may appear in the centre of the group than at the periphery and more blue bodies at the periphery than in the centre. In short, all of the changes described by Prowazek and others as characteristic of trachoma inclusions are seen in the growing cultures of these hemoglobinophilic bacilli.

Similar day-to-day studies were undertaken with the other types of bacteria found most frequently in the eyes diagnosed as trachoma, *e. g.*, streptococci, staphylococci, gonococci, a minute Gram-negative, non-hemoglobinophilic bacillus not before described, found in a few cases of papillary conjunctivitis, xerosis bacillus, and *Micrococcus tetragenus*, but in none of these varieties except the gonococcus were similar changes found in the same marked degree.

The fact that the gonococcus cultures showed such definite appearances similar to the trachoma inclusions led us to make a special study of a series of ophthalmia neonatorum cases.

In all of the inclusion cases where gonococci are found, apparent transition forms between gonococcus and inclusion are very evident, and we find from a further microscopic study of these slides that the inclusions on the whole present certain characteristics different from those found in our series produced, according to our hypothesis, by nests of growing hemoglobinophilic bacilli.

From this comparative study of "inclusions" and cultures we have reached the following conclusion:

In many cases of "papillary conjunctivitis" and a certain number of cases of ophthalmia neonatorum, as well as in a certain number of cases of inflammation of the mucous membranes of other parts of the body (*e. g.*, vagina, urethra), the trachoma inclusions found are due to one or more varieties of hemoglobinophilic bacilli; in a certain number of cases of gonorrheal ophthalmia as well as in gonorrheal inflammation of the mucous membranes of other parts of the body, the trachoma inclusions are due to the gonococcus. According to one of the later

reports of Leber and Prowazek and the reports of Noguchi and Cohen, certain inclusion conjunctivitis cases may be caused by microorganisms other than the two mentioned above.

### THE BORDET-GENGOU BACILLUS (B. PERTUSSIS).

In 1906 Bordet and Gengou announced that they had discovered the etiological factor of whooping-cough to be a small bacillus found in predominating numbers in whooping-cough sputum. To this organism they gave the name *Bacillus pertussis*. Their claim that this bacillus is the real cause of whooping-cough they based upon their results with the complement-fixation test which they had been first to describe.

**Morphology.**—The pertussis bacillus is a short, oval rod, varying in size from about  $0.2\mu$  to  $0.3\mu$  in diameter and from  $0.5\mu$  to  $2\mu$  in length. It occurs singly, sometimes in twos joined at the ends, and very exceptionally in short chains.

**Motility.**—It is non-motile.

**Staining.**—The pertussis bacillus is decolorized by Gram's method. It is stained faintly by the ordinary aniline dyes. Bipolar staining is demonstrated very well by Gram's method and by toluidin blue (p. 78).

**Cultivation.**—The pertussis bacillus grows best at  $35^{\circ}$  C., to  $37^{\circ}$  C. It grows slowly at room temperature.

It is aerobic, facultative anaerobic. When first isolated in pure culture it grows only upon the glycerin-potato-blood-agar, recommended by Bordet and Gengou (p. 105). In later generations it grows more or less capriciously, upon the ordinary culture media.

**Isolation.**—The pertussis bacillus is isolated from sputum with difficulty, owing to the fact that in cultures it is frequently overgrown by other organisms found in the respiratory tract. The sputum should be collected from the patient during the early stage of the disease, best in the first week. The thick grayish portion of the sputum should be selected for the culture. This material is streaked over the surface of a plate of Bordet-Gengou medium. The plates are incubated at  $35^{\circ}$  C. to  $37^{\circ}$  C. In forty-eight hours to three days very minute, discrete, elevated colonies appear. When these colonies occur in abundance, the blood at their periphery, is lighter red. In pure cultures this lightning of the blood is marked and may appear as hemolysis.

**Identification.**—(a) **Differential Diagnosis by Culture.**—There are other bacilli occurring in whooping-cough sputum so closely resembling the pertussis bacillus in morphological and staining characteristics that they cannot be distinguished in smears. These bacilli, however, can be differentiated by their growth upon various culture media. The most important of these organisms is the influenza bacillus, which never grows alone without the presence of hemoglobin in the culture medium. There is frequently found a Gram-negative bacillus which makes a pro-

fuse growth upon all media from the first generation in pure culture. The following table gives the chief points of differentiation:

	Growth on Bordet-Gengou plates.	Bordet-Gengou slants.	Coagulated horse-blood slants.	Glycerin-ascites-agar slants.	Plain agar slants.
<i>B. pertussis</i> . . . .	Lightens the medium.	First generation in pure culture abundant non-spreading moist growth in twenty-four hours.	After several generations abundant tenacious growth in forty-eight hours.	After several generations abundant tenacious growth in forty-eight hours.	After several generations tenacious growth occurs slowly.
<i>B. influenzae</i> . . . .	Darkens the medium.	First generation: delicate growth.	First generation: abundant moist spread in twenty-four hours.	Never grows.	Never grows.
Intermediate group of Gram-negative bacilli.	Markedly lightens the medium.	First generation: abundant moist spread in twenty-four hours.	First generation: profuse moist spread in twenty-four hours.	First generation: profuse moist spread in twenty-four hours.	First generation: profuse moist spread in twenty-four hours.

(b) **By Agglutination.**—Agglutinins are easily obtained in rabbits for the *B. pertussis* and by absorption tests we have shown that all strains tested by us belong to the same species. It is easy therefore to identify any strain isolated. It is not so easy, however, to diagnose a human case of whooping-cough by this method since agglutinins are not produced to any extent in the natural affection.

(c) **Complement-fixation.**—Bordet and Gengou regarded the complement-fixation as the main support for their claim of the specificity of their organism. They report positive complement-fixation in the majority of human cases tested by them. Other investigators, however, have reported negative tests of complement-fixation.

The irregular reports given of the complement-fixation test in human beings may be accounted for by different methods used without making comparative studies and sufficient controls. The question of the best method of making the complement-fixation test is still under experiment (p. 199). Different observers have used different methods of preparing both antigen and serum and have employed different hemolytic systems. In experimental animals, specific antibodies are produced which give a positive complement-fixation test.

**Pathogenicity.**—The specific pathogenicity of the pertussis bacillus for man still lacks proof, though the evidence is strongly in favor of this organism being the cause of pertussis. Several investigators have reported its pathogenicity for monkeys, dogs, rabbits, and guinea-pigs.

Mallory claims that a certain number of bacilli between the cilia of the epithelial cells in the trachea and bronchi constitute the specific lesion. He claims that he has fulfilled Koch's laws by the finding of these bacilli in experimental animals in the same situation and by the recovery of the culture from these animals. He acknowledges, however, that his results in animals are complicated by the fact that these animals

are frequently infected by the *Bacillus bronchisepticus* (accepted as the cause of distemper in dogs) which is morphologically similar to the pertussis bacillus and that it apparently has the same power (as Theobald Smith and others have pointed out) to cling to the cilia of the epithelial cells in the respiratory tract. In human beings Mallory states, judging from the examination of hundreds of controls, only pertussis cases showed this lesion.

The results of treatment by vaccine are given in Part III.

### THE BACILLUS OF SOFT CHANCRE.

This bacillus was first specifically described and obtained in pure culture by Ducrey in 1889. An experimental inoculation is followed in one or two days by a small pustule. This soon ruptures and a small round depressed ulcer is left. About this other pustules and ulcers develop which tend to become confluent. The base of the ulcer is covered with a gray exudate and its edges are undermined. There is no induration such as in the syphilitic chancre. The secretion is seropurulent and very infectious. The process usually extends to the neighboring lymphatics, which become swollen and may result in abscesses. These are known as "buboes."

**Morphology.**—About  $1.5\mu$  long and  $0.4\mu$  thick, growing often in chains and in cultures, sometimes twisted together in dense masses.

It stains best with carbol-fuchsin, and shows polar staining. It is Gram-negative.

**Cultural Characteristics.**—The following method of cultivation has given the best results: Two parts of liquefied agar at  $50^{\circ}$  C. are mixed with one part human, dog, or rabbit blood. The blood from the cut carotid of a rabbit may be allowed to run directly into the agar tube, to which the pus from the ulcerated bubo is then added in proper proportion, and the whole placed in the incubator at  $35^{\circ}$  C. The pus may be obtained by puncture and aspiration from the unbroken ulcer, or if the ulcer is already open it is first painted with tincture of iodine and covered with collodion or sterile gauze. After twenty-four to forty-eight hours, some pus having collected under the bandage, inoculations are made from it. The bacillus grows well also in uncoagulated rabbit-blood serum or in condensation water of blood agar. In twenty-four to forty-eight hours, on the surface of the media, well-developed, shining, grayish colonies, about 1 mm. in diameter may be observed. The colonies remain separate, but they increase in numbers after further transplantation. The best results are obtained when the pus is taken close to the walls of the abscess. Smears show isolated bacilli or short parallel chains with distinct polar staining.

The characteristic soft chancre is produced in man after inoculation of cultures. Animals in general cannot be infected, but positive results have been obtained with monkeys and cats.

The organisms are especially characteristic in the water of condensation from blood agar, the bacilli being thinner and shorter, with

rounded ends; sometimes long, wavy chains are found. In rabbit-blood serum at 37° C. a slight clouding of the medium is produced and small flakes are formed, consisting of short bacilli or moderately long, curved chains, showing polar staining.

The bacillus lives several weeks on blood agar at 37° C., but it soon dies in cultures on coagulated serum. All other ordinary culture media so far tried have given negative results, and even with the media described, development is difficult and often fails entirely.

The chancre bacillus possesses but little resistance to deleterious outside influences. Hence the various antiseptic bandages, etc., used in treatment of the affection soon bring about recovery by preventing the spread of inoculation chancre.

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## CHAPTER XXXI.

### MICROÖRGANISMS BELONGING TO THE HEMORRHAGIC SEPTICEMIA GROUP.

A NUMBER of bacilli of similar characteristics have been described as causing certain infectious diseases of lower animals, marked by the appearance of hemorrhagic areas throughout the body (hemorrhagic septicemia of Hueppe). The bacilli are short, non-motile, non-spore-bearing organisms. They exhibit bipolar staining and are Gram-negative. They do not liquefy gelatin. They are found in rabbit septicemia, fowl cholera, swine plague, and a similar disease in cattle. The bacillus of bubonic plague seems to be closely related to the bacteria of this group.

#### BACILLUS OF CHICKEN CHOLERA.

(*Bacillus Avisepticus*.)

In 1880 Pasteur carried on some fundamental studies on the bacillus of chicken cholera. The bacillus was isolated from a widely disseminated acute disease of fowls and smaller birds.

**Characteristics.**—It is a short ( $0.5-1\mu$  long), non-motile bacillus with marked polar staining. In general, its characteristics are similar to those of other members of this group.

**Pathogenicity.**—Pure cultures are very pathogenic for chickens and rabbits, less so for sheep, pigs, and horses. Chickens are infected even by feeding minute amounts. A septicemia is produced which is rapidly fatal.

#### BACILLUS OF SWINE PLAGUE.

(*Bacillus Suisepiticus*.)

This organism is morphologically and culturally similar to the *B. avisepticus*. It differs in pathogenesis in that it is naturally a disease of swine, characterized by a more or less chronic bronchopneumonia followed by septicemia. The gastro-intestinal tract is not markedly affected. The disease is generally fatal in young pigs.

The "bacillus of hog cholera" (see p. 355) may often be found as a mixed infection with the *B. suisepiticus*.

#### BACILLUS OF BUBONIC PLAGUE (BACILLUS PESTIS).

Historically we can trace the bubonic plague back to the third century. In Justinian's reign a great epidemic spread over the Roman empire and before it terminated destroyed in many portions of the country nearly 50 per cent. of the people. The fourteenth century saw the whole



of Europe stricken with this "black death." Europe and America have of late been practically free, but in India the disease still breaks out in all its horrors so that at the present time over 500,000 persons die annu-

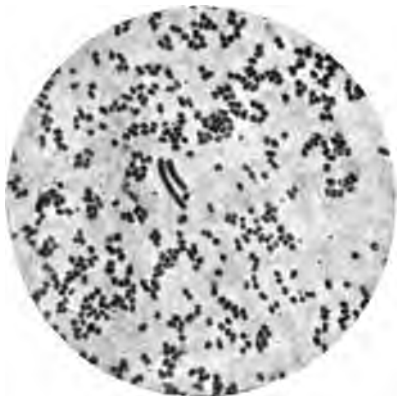


FIG. 146.—*Bacillus pestis* from agar culture.  $\times 1100$  diameters.



FIG. 147.—*Bacillus pestis* from bouillon culture.  $\times 1100$  diameters.

ally from it. Among the most fatal forms of infection is that of the lungs. Pneumonic cases are not only very serious, but they readily spread the infection. The bacillus exciting the disease was discovered simultaneously by Kitasato and Yersin (1894) during an epidemic of the bubonic plague in China. It is found in large numbers in the seropurulent fluid

from the recent buboes characteristic of this disease and in the lymphatic glands; more rarely in the internal organs except in pneumonic cases when the lungs and sputum contain immense numbers. It occurs in the blood in acute hemorrhagic cases and shortly before death. It also occurs in malignant cases in the feces of men and animals. The bacillus, as we have stated, is closely allied to the hemorrhagic septicemia group.

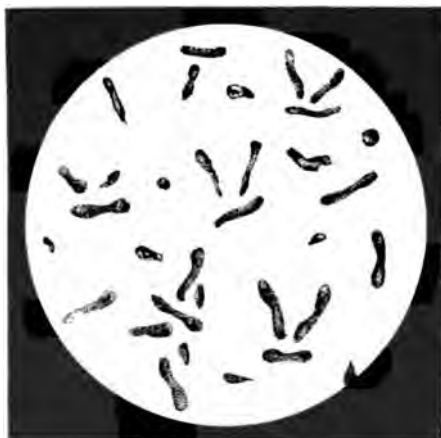


FIG. 148.—Involution forms on salt agar. (Kolle and Wassermann.)

**Morphology.**—The bacilli in smears from acute abscesses or infected tissues are, as a rule, short, thick rods with rounded

ends. The central portion of the bacillus is slightly convex. The bacilli are mostly single or in pairs. Bacilli in short chains occur at times. The length of the bacilli varies, but on the average is about

1.6 $\mu$  (1.5 $\mu$  to 1.7 $\mu$ ), breadth 0.5 $\mu$  to 0.7 $\mu$ . Besides the usual oval form, the plague bacillus has many exceptional variations which are characteristic of it. In smears, especially from old buboes, one looks for long bacilli with clubbed ends (similar to involution forms (Fig. 148), yeast-like forms, and bladder shapes. Some of these stain with difficulty.

**Staining.**—They stain readily with the ordinary aniline dyes, and especially well with methylene blue, the ends being usually more deeply colored than the central portion; they do not stain by Gram's method.

**Biology.**—An aerobic, non-motile bacillus. Grows best at 30° to 35° C. Does not form spores. Grows on the usual culture media, which should have a slightly alkaline reaction. Does not liquefy gelatin. Grows well on *blood-serum* media. It grows rapidly on *glycerin agar*, forming a grayish-white surface growth. The bacilli appear, as a rule, as short, plump, oval bacilli, but a few present elongated thread forms which are very characteristic. In *bouillon* which is kept undisturbed a characteristic appearance is produced, the culture medium remaining clear while a pellicle forms on the surface from which projections sprout downward (stalactite formation) toward a granular or grumous deposit which forms on the walls and on the bottom of the tube. In *bouillon* and most fluid media the growth is in the form of short or medium chains of very short, oval bacilli, which look almost like streptococci. It does not coagulate milk, but produces a slight acidity. It produces no indol in peptone media.

**Pathogenicity.**—Plague is a rodent disease transmissible to man. The most important rodent is the rat. Mice may become infected. Other rodents may be infected and be the means of maintaining the disease in endemic areas, as the marmot in Thibet or the ground squirrel in California. The tarbagan was primary source of infection in the epidemic of pneumonic plague in Manchuria (1910–11). Other species of rodents may later be found to be reservoirs of the disease.

These animals as well as guinea-pigs are easily infected artificially by feeding or application of the bacilli to the mucous membranes or to the skin. In the last, infection is sure to follow if a slight puncture or scratch is present. Monkeys and rabbits can also be infected. The bacilli may lose their virulence after prolonged artificial cultivation.

In *rodents* the disease may be either acute or chronic. A septicemia develops as a terminal event and fleas feeding at this time become infected. In *man*, the disease is bubonic pneumonic or septicemic in type. Septicemia is the usual mode of termination of the bubonic and pneumonic types of the disease.

The diagnosis of natural infection in rats is made macroscopically, although occasionally the disease does occur without evident lesions. In acute plague the engorgement of the subcutaneous bloodvessels and the diffuse pink color of the subcutaneous tissues and muscles is strongly diagnostic. The superficial lymph nodes are very much enlarged and frequently surrounded by edema or hemorrhagic areas. The spleen is very much enlarged and soft. The liver is mottled with small hemorrhages and yellowish punctate areas of necrosis. Generally, there is an

excess of fluid in the pleural cavity. In the more chronic disease, abscesses of the peripheral lymph nodes or more commonly mesenteric or splenic purulent or caseous foci are found. Frequently the rats submitted for examination are badly decomposed but pure cultures of the plague bacillus may be readily obtained by applying the material from the lesions to the freshly shaven abdomen of a guinea-pig. The plague bacilli penetrate the skin through the slight scarification due to shaving, whereas the other bacteria do not and a general infection results.

**Epidemiology.**—The disease is maintained in the rodents. Although direct contagion may occur, the most important mode of transfer is by rodent fleas. The bacilli taken up with infected blood multiply in the digestive tract of the flea but the mouth parts seem not to remain infected. Fleas may remain infected for weeks or even one or two months if the

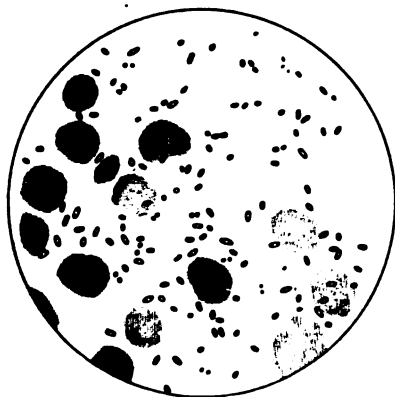


FIG. 149.—Bacilli in smear from acutely inflamed gland.

temperature is low. The mechanism of infection is probably the deposition of infected feces during the act of feeding or regurgitation of infected blood from a previous meal. Plague bacilli can invade the tissues through a flea bite, thus if a non-infected flea is allowed to bite a rat and a drop of plague cultures be placed on the bite infection results.

Bubonic plague in man is due to transfer of infection by means of fleas. The evidence for this is only indirect based on the facts that rodent fleas will bite man, the parallelism between outbreaks of plague in man with the prevalence of the disease in rats and the seasonal rise in the number of fleas, the lymphatic involvement suggesting primary skin infection, etc. The pneumonic type may develop after infection by fleas due to blood invasion and pulmonary localization. Should this occur an outbreak of pneumonic plague may develop due to man-to-man contagion. This is most likely to occur during cold weather and under conditions of close contact. This form of disease is extremely contagious, with a mortality of 90 per cent. or more. The mortality of bubonic plague varies from 30 to 90 per cent.

Other insects than fleas may possibly be a factor in infection, for example bed-bugs. It is possible that human vermin were a factor in the widespread outbreaks of "black death" during the middle ages.

**Immunity.**—Like typhoid infection, a single attack of the plague bacillus protects, with rare exception, from a second infection. Yersin, Calmette, and Borrel have succeeded in immunizing animals against the bacillus of bubonic plague by the intravenous or intraperitoneal injection of dead cultures, or by repeated subcutaneous inoculation. They also succeeded in immunizing rabbits and horses, so that the serum afforded protection to small animals, after subcutaneous injection, of virulent cultures, and even cured those which had been inoculated, if administered within twelve hours after injection. The serum has considerable antitoxic as well as bactericidal properties. It also contains specific agglutinins which may be made use of in diagnosis. For use of vaccine and serum see Part III.

**Duration of Life Outside of the Body.**—In cultures protected from the air and light the plague bacilli may live ten years or more (Wilson). In the bodies of dead rats they may live for two months. In sputum from pneumonic cases the bacilli lived ten days. Upon sugar sacks, food, etc., they may live six to fifteen days.

**Resistance to Deleterious Influences.**—The bacilli resemble the colon bacilli in their reaction to heat and disinfectants.

**Bacteriological Diagnosis.**—Material is obtained in bubonic plague by puncture or incision of a lymph node, in pneumonic plague the sputum is employed. Direct smears if plague-like bacilli are present are valuable in making a rapid presumptive diagnosis, cultures and cutaneous inoculation of guinea-pigs should also be made. Antemortem blood cultures are frequently positive especially late in the disease. In postmortem examinations the heart blood and spleen should be examined. Wherever the material is badly contaminated or even decomposed the cutaneous method of inoculation of guinea-pigs should be resorted to. For rodents see p. 423.

**Plague-like Disease in Rodents.**—McCoy and Chapin (1912) found an organism (*Bacillus tularensis*) in a disease of Californian ground squirrels, which show lesions similar to those of plague. The bacilli have been cultivated by McCoy and Chapin on an egg-yolk medium. Wherry and Lamb<sup>1</sup> report two cases of conjunctivitis and lymphadenitis in man due to this bacillus, as well as an epidemic among wild rabbits.

<sup>1</sup> Jour. Am. Med. Assn., 1914, lxiii, 2041.

## CHAPTER XXXII.

### THE ANTHRAX BACILLUS.

ANTHRAX is an acute infectious disease which is very prevalent among animals, particularly sheep and cattle. Geographically and zoologically it is the most widespread of all infectious disorders. It is much more common in Europe and in Asia than in America. The ravages among herds of cattle in Russia and Siberia and among sheep in certain parts of France, Hungary, Germany, Persia, and India are not equalled by any other animal plague. Local epidemics have occasionally occurred in England, where it is known as splenic fever. In this country the disease is rare. In infected districts the greatest losses are incurred during the hot months of summer.

The disease also occurs in man as the result of infection either through the skin, the intestines, or in rare instances through the lungs. It is found in persons whose occupations bring them into contact with animals or animal products, as stablemen, shepherds, tanners, butchers, and those who work in wool and hair. Two forms of the disease have been described—the external anthrax, or magignant pustule, and the internal anthrax, of which there are intestinal and pulmonary forms, the latter being known as “wool-sorters’ disease.”

Owing to the fact that anthrax was the first infectious disease which was shown to be caused by a specific microorganism, and to the close study which it received in consequence, this disease has probably contributed more to our general knowledge of bacteriology than any other infectious malady.

Davaine, in 1863, announced to the French Academy of Sciences the results of his inoculation experiments, and asserted the etiological relations of the microorganism to the disease, with which his investigation showed it to be constantly associated. Pollender corroborated this statement. In 1877 Koch, Pasteur, and others established its truth by obtaining the bacillus in pure cultures, and showing that the inoculation of these cultures produced anthrax in susceptible animals as certainly as did the blood of an animal recently dead from the disease.

**Morphology.**—Slender, cylindrical, non-motile rods, having a breadth of  $1\mu$  to  $1.25\mu$ , and ranging from  $2\mu$  or  $3\mu$  to  $20\mu$  or  $25\mu$  in length. Sometimes short, isolated rods are seen, and, again, shorter or longer chains or threads made up of several rods joined end-to-end. In suitable culture media very long, flexible filaments may be observed, which are frequently united in twisted or plaited cord-like bundles. (See Figs. 151 and 152.) These filaments in hanging-drop cultures, before the development of spores, appear to be homogeneous or nearly so;

but in stained preparations they are seen to be composed of a series of rectangular, deeply stained segments. When obtained directly from the blood of an infected animal the free ends of the rods are slightly rounded, but those coming in contact with one another are quite square. In cultures the ends are seen to be a trifle thicker than the body of the cell and somewhat concave, giving the appearance of joints of bamboo. At one time much stress was laid upon these peculiarities as distinguishing marks of the anthrax bacillus; but it has been found that they are the effects of artificial cultivation and not necessarily characteristic of the organism under all conditions. The bacillus is inclosed in a transparent envelope or capsule, which in stained preparations (from albuminous material) may be distinguished by its taking on a lighter stain than the deeply stained rods which it surrounds.



FIG. 150.—Anthrax bacillus.  $\times 400$  diameters. Agar culture.

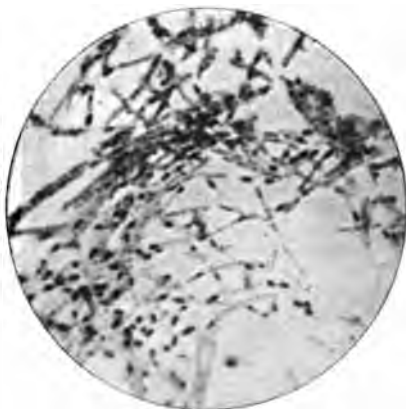


FIG. 151.—Spores heavily stained (in specimen red). Bodies of disintegrating bacilli faintly stained (in specimen blue).  $\times 1000$  diameters.

**Spore Formation.**—Under favorable conditions in cultures spores are developed in the bacilli. These spores are elliptic in shape and about one and a half times longer than broad. They first appear as small, refractive granules distributed at regular intervals, one in each rod. As the spore develops the mother-cell becomes less and less distinct, until it disappears altogether, the complete oval spore being set free by its dissolution. (See Fig. 151 and Plate III, Fig. 22). Irregular sporulation sometimes takes place, and occasionally there is no spore formation, as in varieties of non-spore-bearing anthrax.

Sporeless varieties have been produced artificially by cultivating the typical anthrax bacillus under certain conditions, among which may be mentioned the addition of antiseptics, as carbolic acid, and of continued high temperature ( $43^{\circ}$  C.). Varieties differing in their pathogenic power may also be produced artificially. Pasteur produced an "attenu-

ated virus" by keeping his cultures for a considerable time before replanting them upon fresh soil.

Anthrax cultures containing spores retain their vitality for years; in the absence of spores the vitality is much more rapidly lost. When grown in liquids rich in albumin the bacilli attain a considerable degree of resistance; thus dried anthrax blood has been found to retain its virulence for sixty days, while dried bouillon cultures only did so for twenty-one days. Dried anthrax spores may be preserved for many years without losing their vitality or virulence. They also resist a comparatively high temperature. Exposed in dry air they require a temperature of 140° C. maintained for three hours to destroy them; but suspended in a liquid they are destroyed in four minutes by a temperature of 100° C.

**Staining.**—The anthrax bacillus *stains* readily with all the aniline colors, and also by Gram's method, when not left too long in the decolorizing solution. In sections good results may be obtained by the employment of Gram's solution in combination with carmine, but when only a few bacilli are present this method is not always reliable, as some of the bacilli are generally decolorized.

**McFadyean-Heine Methylene-blue Reaction.**—In imperfectly fixed film preparations (pass through flame three times in a second with film side up) the capsule disintegrates. When a solid film is stained for a few seconds in an old solution of methylene blue, washed in water and dried with filter paper, the bacteria are surrounded by a varying amount of a reddish-purple amorphous or fixed granular deposit. McFadyean says this does not occur with other morphologically similar bacteria.

**Biology.**—The anthrax bacillus grows easily in a variety of nutrient media at a temperature from 18° to 43° C., 37° C. being the most favorable temperature. Under 12° C. no development takes place as a rule, though by gradually accustoming the bacillus to a lower temperature it may be induced to grow under these conditions. Under 14° C. and above 43° C. spore formation ceases. The lower limit of growth and of sporulation is of practical significance in determining the question whether development can occur in the bodies of animals dead from anthrax when buried at certain depths in the earth. Kitasato has shown that at a depth 1.5 meters the earth in July has a temperature of 15° C. at most, and that under these conditions a scanty sporulation of anthrax bacilli is possible, but that at a depth of 2 meters sporulation no longer occurs. The anthrax bacillus is *aërobic*—that is, its growth is considerably enhanced by the presence of oxygen—but it grows also under *anaërobic* conditions, as is shown by its growth at the bottom of the line of puncture in stick cultures in solid media; but under these conditions it no longer produces the peptonizing ferment which it does with free access of air. Furthermore, the presence of oxygen is absolutely necessary for the formation of spores, while carbonic acid gas retards sporulation. This explains, perhaps, why sporulation does not take place within the animal body either before or after death.

It is also capable of leading a saprophytic existence. The bacillus is non-motile.

**Growth in Gelatin.**—In *gelatin-plate cultures*, at the end of twenty-four to thirty-six hours at 24° C., small, white, opaque colonies are developed, which, under a low-power lens, are seen to be dark gray in the centre and surrounded by a greenish, irregular border, made up of wavy filaments. As the colony develops on the surface of the gelatin these wavy filaments spread out, until finally the entire colony consists of a light gray, tangled mass, which has been likened to a Medusa head (Fig. 152).

At the same time the gelatin begins to liquefy, and the colony is soon surrounded by the liquefied medium, upon the surface of which it floats as an irregular, white pellicle. In *gelatin-stick cultures* at first, development occurs along the line of puncture as a delicate white thread, from which irregular, hair-like projections soon extend perpendicularly into the culture medium, the growth being most luxuriant

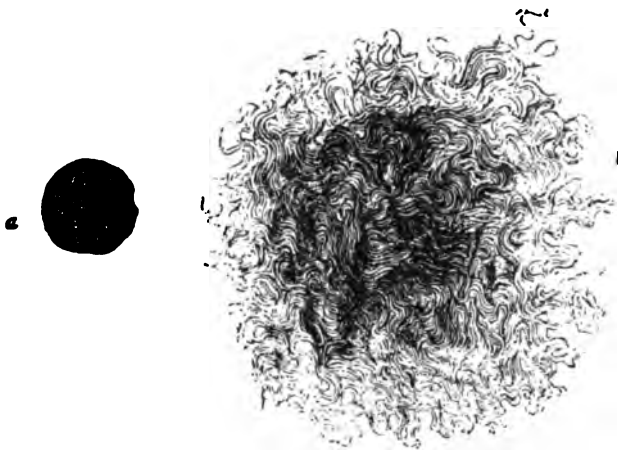


FIG. 152.—Colonies of *Bacillus anthracis* upon gelatin plates: *a*, at the end of twenty-four hours; *b*, at the end of forty-eight hours.  $\times 80$ . (F. Flüge.)

near the surface, but continuing also below. At the end of two or three days' liquefaction of the medium commences at the surface and gradually progresses downward.

**Growth on Agar.**—The growth on *agar-plate cultures* in the incubator at 37° C. is similar to that on gelatin, and is still more characteristic and beautiful in appearance. A grayish-white layer is formed on the surface within twenty-four hours, which spreads rapidly and is seen to be made up of interlaced threads.

**Growth in Bouillon.**—The growth is characterized by the formation of flaky masses, which sink as a sediment to the bottom of the tube, leaving the supernatant liquid clear.

**Pathogenesis.**—The anthrax bacillus is pathogenic for cattle, sheep (except the Algerian race), horses, swine, mice, guinea-pigs, and rabbits. Rats, cats, dogs, chickens, owls, pigeons, and frogs are but little susceptible to infection. Small birds—the sparrow particularly—are



somewhat susceptible. Man, though subject to local infection and occasionally to internal forms of the disease, is not as susceptible as some of the lower animals.

In susceptible animals the anthrax bacillus produces a true septicemia. Among test animals mice are the most susceptible, succumbing to very minute injections of a slightly virulent virus; next guinea-pigs, and then rabbits, both of these animals dying after inoculation with virulent bacilli. Infection is most promptly produced by introduction of the bacilli into the circulation or the tissues, but inoculation by contact with wounds on the skin also causes infection. It is difficult to produce

infection by the ingestion even of spores; but it may readily be caused by inhalation, particularly of spores.

Subcutaneous injections of these susceptible animals results in death in from one to three days. Comparatively little local reaction occurs immediately at the point of inoculation, but beyond this there is an extensive edema of the tissues. Very few bacilli are found in the blood in the larger vessels, but in the internal organs, and especially in the capillaries of the liver, the kidneys, and the lungs, they are present in great numbers. In some places, as in the glomeruli

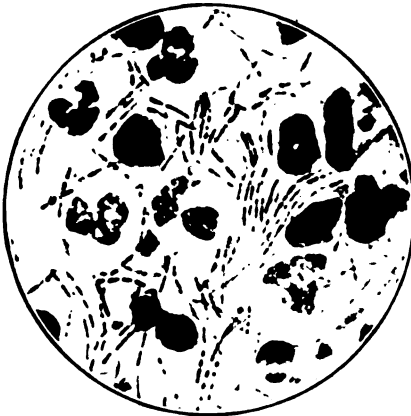


FIG. 153.—Section of liver from mouse dead of anthrax septicemia.  $\times 1000$  diameters. (From Itzerott and Niemann.)

of the kidneys, the capillaries will be seen to be stuffed full of bacilli, and hemorrhages, probably due to rupture of capillaries by the mechanical pressure of the bacilli which are developing within them, may occur. The pathological lesions in animals infected by anthrax are not marked except in the spleen, which, as in other forms of septicemia, is greatly enlarged.

**Occurrence in Cattle and Sheep.**—Cattle and sheep are affected chiefly with the intestinal form of anthrax, infection in these animals commonly resulting from the ingestion of food containing spores. The bacillus itself, in the absence of spores, is quickly destroyed by the gastric juice. The disease usually takes a rapid course, and the mortality is high—70 to 80 per cent. The pathological lesions consist of numerous ecchymoses, enlargement of the lymphatic glands, serous, fatty, and hemorrhagic infiltration of the mediastinum and mesentery, of the mucous membranes of the pharynx and larynx, and particularly of the duodenum, great enlargement of the spleen, and parenchymatous changes in the lymphatic organs. The blood is very dark and tar-like. Bacilli are present, especially in the lymph spaces, in enormous masses.

Sheep are also subject to external anthrax, infection taking place by way of the skin; cattle are seldom infected in this way. At the

point of inoculation there develops a hard, circumscribed boil—the so-called anthrax carbuncle; or there may be diffuse edema with great swelling of the parts. When death occurs the appearances are similar to those in intestinal anthrax, except that the duodenum is usually less affected; but in all cases metastasis occurs in various parts of the body, brought about, no doubt, by previous hemorrhages.

**Occurrence in Man.**—The disease does not occur spontaneously in man, but always results from infection, either through the skin, the intestines, or occasionally by inhalation through the lungs. It is usually produced by cutaneous infection through inoculation of exposed surfaces—the hands, arms, or face. Infection of the face or neck would seem to be the most dangerous, the mortality in such cases being 26 per cent., while infection of the extremities is rarely fatal.

External anthrax in man is similar to this form of the disease in animals. There are two forms: malignant pustule or carbuncle, and, less commonly, malignant anthrax edema.

*In malignant pustule*, at the site of inoculations, a small papule develops, which becomes vesicular. Inflammatory induration extends around this, and within thirty-six hours there is a dark, brownish eschar in the centre, at a little distance from which there may be a series of small vesicles. The brawny induration may be extreme. There may also be considerable edema of the parts. In most cases there is no fever; or the temperature at first rises rapidly and the febrile phenomena are marked. Death may take place in from three to five days. In cases which recover the symptoms are slighter. In the mildest form there may be only slight swelling.

*Malignant anthrax edema* occurs in the eyelids, and also in the head and neck, sometimes the hand and arm. It is characterized by the absence of the papule and vesicle forms, and by the most extensive edema. The edema may become so intense that gangrene results; such cases usually prove fatal.

The bacilli are found on microscopic examination of the fluid from the pustule shortly after infection; later the typical anthrax bacilli are often replaced by involution forms. In this case resort may be had to cultures, animal inoculation, or examination of sections of the extirpated tumor. The bacilli are not present in the blood until just before death. Along with the anthrax bacilli pyogenic cocci are often found in the pustule penetrating into the dead tissue.

*Internal anthrax* is much less common in man; it does, however, occur now and then. There are two forms of this: the intestinal form, or mycosis intestinalis, and the pulmonic form, or wool-sorters' disease.

*Intestinal anthrax* is caused by infection through the stomach and intestines, and results probably from the eating of raw flesh or unboiled milk of diseased animals. That the eating of flesh from infected animals is comparatively harmless is shown by Gerlief, who states that of 400 persons who were known to have eaten such meat not one was affected with anthrax. On the other hand, an epidemic of anthrax was produced among wild animals, according to Jansen, by feeding them on infected

horse flesh. It is evident, therefore, that there is a possibility of infection being caused in this way. The recorded cases of intestinal anthrax in man have occurred in persons who were in the habit of handling hides, hair, etc., which were contaminated with spores; in those who were conducting laboratory experiments, and rarely it has been produced by the ingestion of food, such as raw ham and milk. The symptoms produced in this disease are those of intense poisoning, chill, followed by vomiting, diarrhea, moderate fever, and pains in the legs and back. The pathological lesions are similar to those described in animals.

*Wool-sorters' disease*, or pulmonic anthrax, is found in large establishments in which wool and hair are sorted and cleansed, and caused by the inhalation of dust contaminated with anthrax spores. The attack comes on with chills, prostration, then fever. The breathing is rapid, and the patient complains of pain in the chest. There may be a cough and signs of bronchitis. The bronchial symptoms in some instances are pronounced. Death may occur in from two to seven days. The pathological changes produced are swelling of the glands of the neck, the formation of foci of necrosis in the air passages, edema of the lungs, pleurisy, bronchitis, enlargement of the spleen, and parenchymatous degenerations.

**Prophylaxis against Anthrax Infection.**—Numerous investigations have been undertaken with the object of preventing infection from anthrax. The efforts of Pasteur to effect immunity in animals by preventive inoculations of "attenuated virus" of the anthrax bacillus opened a new field of productive original research. Following in his wake many others have devised methods of immunization against anthrax infection; but the one adopted by Pasteur, Chamberland, and Roux has alone been practically employed on a large scale. According to these authors, two anthrax cultures of different degrees of virulence attenuated by cultivation at 42° to 43° C., are used for inoculation. Vaccine No. 1 kills mice, but not guinea-pigs; vaccine No. 2 kills guinea-pigs, but not rabbits. The animals to be inoculated—viz., sheep and cattle—are first given a subcutaneous injection of one to several tenths of a cubic centimeter of a four-day-old bouillon culture of vaccine No. 1; after ten to twelve days they receive a similar dose of vaccine No. 2. Prophylactic inoculations given in this way have been widely employed with apparently good results.

**Serum Treatment.**—The serum of immunized animals has been used in Italy with apparently some good results. In this country Eichhorn has reported good results.

**Bacterial Cultures for Diagnosis.**—The detection of the anthrax bacillus is ordinarily not difficult, as this organism presents morphological, biological, and pathogenic characteristics which distinguish it from all other bacteria. In the later stages of the disease, however, the bacilli may be absent or difficult to find, and cultivation on artificial media and experimental inoculation in animals are not always followed by positive results. Even in sections taken from the extirpated pustule it is sometimes difficult to detect the bacilli. In such cases only a

probable diagnosis of anthrax can be made. It should be remembered that the bacilli are not found in the blood until shortly before death, and then only in varying quantity; thus blood examinations often give negative results, though the bacilli may be present in large numbers in the spleen, kidneys, and other organs of the body. The suspected material should be streaked over nutrient agar in Petri plates and inoculated in mice.

**Differential Diagnosis.**—Among other bacteria which may possibly be mistaken for anthrax bacilli are *Bacillus subtilis* and the bacillus of malignant edema. The former is distinguished by its motility, by various cultural peculiarities, and by being non-pathogenic. The latter differs from the anthrax bacillus in form and motility, in being decolorized by Gram's solution, in being a strict anaërobe, and in various pathogenic properties. (See also description in chapter on Water Analysis.)

The diagnosis of internal anthrax in man is by no means easy, unless the history points definitely to infection in the occupation of the individual. In cases of doubt cultures should be made and inoculations performed in animals.

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## CHAPTER XXXIII.

### ANAEROBIC BACILLI.

IN this chapter we are grouping several species of bacteria which have in common only the characteristic that they cannot grow in pure cultures in the presence of oxygen. Those of most interest to us are *B. tetani*, *B. (anthracis) symptomatici*, *B. (edematis) maligni*, *B. welchii*, *B. botulinus* and *B. fusiformis*.

#### THE BACILLUS AND THE BACTERIOLOGY OF TETANUS.

Tetanus is a disease which is characterized by a gradual onset of general spasms of the voluntary muscles, commencing in both man and the horse most often in the muscles of the jaw and neck, and extending in severe cases to the muscles of the body. The disease is usually associated with a wound received from four to fourteen days previously.

Tetanus has been reported for many centuries. The writings of Hippocrates clearly describe the symptoms. In 1884 Nicolaier, under Flügge's direction, produced tetanus in mice and rabbits by the subcutaneous inoculation of particles of garden earth. The Italians, Carle and Rattone, had just before demonstrated that the pus of an infected wound from a person attacked with tetanus could produce the same disease in rabbits. Finally, Kitasato, in 1889, obtained the bacillus of tetanus in pure culture and described his method of obtaining it and its biological characters.

**Occurrence in Soil, etc.**—The tetanus bacillus occurs widely throughout the world as a common inhabitant of the soil, especially in places where manure has been thrown, being abundant in many localities not only in the superficial layers, but also at the depth of several feet. It has been found in many different substances and places—in hay dust, in horse and cow manure (its normal habitat is the intestine of the herbivora), in the mortar of old masonry, in the dust from horses' hair, and in the dust in rooms of houses, barracks, and hospitals.

The tetanus bacilli are more numerous in certain localities than in others—for example, some parts of Long Island and New Jersey have become notorious for the number of cases of tetanus caused by small wounds—and they are fairly common in New York City. As a rule they are more abundant in regions where the temperature is high. In some islands and countries in the tropics cases of puerperal tetanus and tetanus in the newborn are very frequent. Tetanus bacilli are found in the intestines of about 15 per cent. of horses and calves living in the vicinity of New York City. They are also present to a somewhat less extent in the intestines of other animals and of man.

**Morphology.**—From young gelatin cultures the bacilli appear as motile, slender rods, with rounded ends,  $0.3\mu$  to  $0.5\mu$  in diameter by  $2\mu$  to  $4\mu$  in length, usually occurring singly, but, especially in old cultures, often growing in long threads. They form round or nearly round spores, thicker than the cell (from  $1\mu$  to  $1.5\mu$  in diameter), occupying one of its extremities and giving to the rods the appearance of small pins (Fig. 154).

**Staining.**—It is stained with the ordinary aniline dyes, and is not decolorized by Gram's method. The spores are readily stained and may be demonstrated by double-staining with Ziehl's method. The flagella are fairly easily stained on freshly developed bacilli taken from cultures which have been at short intervals several times transplanted.

**Biology.**—An *anaërobic, liquefying, moderately motile* bacillus. It has abundant peritrichic flagella. During the period of spore formation it is not motile. It grows slowly at temperatures from  $20^{\circ}$  to  $24^{\circ}$  C., and best at  $38^{\circ}$  C., when, within twenty-four to thirty hours it forms spores. At temperatures of  $20^{\circ}$  to  $24^{\circ}$  C., spores form in from six to ten days. It will not grow in pure culture in the presence of oxygen, but grows well in an atmosphere of hydrogen gas. If planted with certain other bacteria the tetanus bacillus grows luxuriantly in the presence of oxygen.

**Growth in Media.**—The bacillus of tetanus grows anaërobically in nutrient gelatin and agar of a slightly alkaline reaction. The addition to the media of 1.5 per cent. of glucose causes the development to be more rapid and abundant. It also grows abundantly in alkaline bouillon in an atmosphere of hydrogen. On *gelatin plates* the colonies develop slowly; they resemble somewhat the colonies of the *Bacillus subtilis*, and have a dense, opaque centre surrounded by fine, diverging rays. Liquefaction takes place more slowly, however, than with *Bacillus subtilis*, and the resemblance to these colonies is soon lost.

The colonies on *agar* are quite characteristic. To the naked eye they present the appearance of light, fleecy clouds; under the microscope, a tangle of fine threads.

The *stab cultures in gelatin* exhibit the appearance of a cloudy, linear mass, with prolongations radiating into the gelatin from all sides (arborescent growth). Liquefaction takes place slowly, generally with the production of gas. In *stab cultures in agar* a growth occurs presenting the appearance of a miniature pine tree. *Alkaline bouillon* is rendered somewhat turbid by the growth of the tetanus bacillus. In all cases a production of gas results, accompanied by a characteristic and very disagreeable odor. It develops in *milk* without causing coagulation.

**Resistance of Spores to Deleterious Influences.**—The spores of the tetanus bacillus are very resistant to outside influences; in a desiccated condition they may retain their vitality for several years, and



FIG. 154.—Tetanus bacilli with spores distending ends.  $\times 1100$  diameters.

are not destroyed in two and a half months when present in putrefying material. They withstand an exposure of one hour at 80° C., but are usually killed by an exposure of ten minutes at 105° C. to live steam. They resist the action of 5 per cent. carbolic acid for ten hours. A 5 per cent. solution of carbolic acid, however, to which 0.5 per cent. of hydrochloric acid has been added, destroys them in two hours. They are killed when acted upon for three hours by bichloride of mercury (1 to 1000), and in thirty minutes when 0.5 per cent. HCl is added to the solution. Silver nitrate solutions destroy the spores of average resistance in one minute in 1 per cent. solution and in about five minutes in 1 to 1000 solution.

With regard to the persistence of tetanus spores upon objects where they have found a resting place, Henrijean reports that by means of a splinter of wood which had once caused tetanus he was able after eleven years again to cause the disease by inoculating an animal by means of the same splinter.

**Isolation of Pure Cultures.**—The growth of the tetanus bacillus in the animal body is comparatively scanty, and is usually associated with that of other bacteria; hence the organism is difficult to obtain in pure culture. The method of procedure which is most successful, consists in inoculating the tetanus-bearing material (pus or tissue from the inoculation wound) into tubes of freshly sterilized slightly alkaline nutrient agar or fermentation tubes of glucose bouillon to each of which a piece of fresh sterile tissue has been added, and incubating at 37° C. After the tetanus spores have formed as shown in microscopic preparations from the sediment about the piece of tissue, heat for one-half hour at 80° C. to destroy the associated bacteria and subinoculate in broth and make plates. If the tetanus bacilli are the only spore-bearing bacteria present, pure cultures are readily obtained; when other spore-bearing anaerobic bacteria are present, the isolation of a pure culture may be a matter of difficulty, but even then the presence of tetanus toxin in the culture fluid, shown by the inoculation of animals, will indicate the presence of tetanus bacilli.

**Pathogenesis.**—In mice, guinea-pigs, rabbits, horses, cattle, goats and a number of other animals inoculations of pure cultures of the tetanus bacillus cause tetanus after an incubation of from one to four days. In the smaller animals tetanus usually develops first in the muscles nearest the point of inoculation. A mere trace of an old culture—only as much as remains clinging to a platinum needle—is often sufficient to kill very susceptible animals like mice and guinea-pigs. Other animals require a larger amount. Rats and birds are but little susceptible, and fowls scarcely at all. These never develop tetanus from natural infection. It is a remarkable fact that an amount of toxin sufficient to kill a hen would suffice to kill 500 horses. It is estimated that if 1 gram of horse requires 1 part of toxin to kill, then 1 gram of guinea-pig requires 6 parts, 1 of mouse 12, of goat 24, of dog 500, of rabbit 1500, of cat 6000, of hen 360,000. Horses frequently develop tetanus after injuries or operations. Cultures from different cases vary in their toxicity. On the inoculation of less than a fatal dose in test animals a local tetanus may be produced, which lasts for days and weeks and then ends in recovery. On killing the animal there is found at autopsy, just at the point of inoculation, a hemorrhagic spot, and no changes other than

these here or in the internal organs. A few tetanus bacilli may be detected locally with great difficulty, often none at all; possibly a few may be found in the region of the neighboring lymphatic glands and even in the blood. From this scanty occurrence of bacilli the conclusion has been reached that the bacilli of tetanus, when inoculated in pure culture, do not multiply to any great extent in the living body, but only produce lesions through the absorption of the poison which they develop at the point of infection. It has been found that pure cultures of tetanus, after the germs have sporulated and the toxins been destroyed by heat, can be injected into animals without producing tetanus. But if pathogenic streptococci or staphylococci or even non-pathogenic organisms are injected simultaneously with the spores, or if there is an effusion of blood at the point of injection, or if there was a previous or later bruising of the tissues, the animals surely die of tetanus.

**Natural Infection.**—Here the infection may be considered as probably produced by the bacilli in their spore state, and the conditions favoring infection are almost always present. A wound of some kind has occurred, penetrating at least through the skin, though perhaps of a most trivial character, such as might be caused by a dirty splinter of wood, and the bacilli or their spores are thus introduced from the soil in which they are so widely distributed. If in any given case, the tissues being healthy, the ordinary saprophytic germs are killed by proper disinfection at once, a mixed infection does not take place, and tetanus will not develop. If, however, other bacteria, especially pathogenic or putrefactive types accompany the tetanus bacilli, or if the tissues infected be bruised or lacerated, the spores may develop and produce the disease. Gelatin and catgut are occasionally found to contain tetanus spores.

**Tetanus in Man.**—Man and almost all domestic animals are subject to tetanus. It is a comparatively rare disease in the United States, during times of peace, except after the Fourth of July celebration, when a considerable number of cases develop. Injuries causing crushing of tissues with accompanying contamination are the most likely to be followed by tetanus. Until immunization became the practice more than one hundred persons yearly developed tetanus after blank cartridge wounds. On examination of an infected individual very little local evidence of the disease can be discovered. Generally at the point of infection, if there is an external wound, some pus is to be seen, in which, along with numerous other bacteria, tetanus bacilli or their spores may be found. Although rather deep wounds are usually the seat of infection, at times such superficial wounds as an acne pustule or a vaccination may give the occasion for infection. In rare cases tetanus has developed from the infection of necrotic mucous membranes as in diphtheria. Not only undoubted traumatic tetanus, but also all the other forms of tetanus, are now conceded to be produced by the tetanus bacillus—puerperal tetanus, tetanus neonatorum, and idiopathic tetanus. In tetanus neonatorum infection is introduced through the navel, in puerperal tetanus through the inner surface of the uterus.



It should be borne in mind that when there is no external and visible wound there may be an internal one. The lesions in the nervous system are still obscure. Congestion, cellular exudate into the perivascular spaces, and chromatolysis of the ganglion cells are common.

**Toxins of the Tetanus Bacillus.**—It is evident from the localization of the tetanus bacilli almost wholly at the point of inoculation and their moderate multiplication at this point that they exert their action through the production of powerful toxins. These toxins are named, according to their action, the tetanospasmin and the tetanolysin. The first only is of importance. One ten-thousandth of a cubic centimeter of the filtrate of an eight-day bouillon culture of a fully virulent bacillus is sufficient to kill a mouse. The purified and dried tetanus toxin prepared by Brieger and Cohn was surely fatal to a 15-gram mouse in a dose of 0.000005 gram. The toxin is precipitated by saturating the broth with ammonium sulphate and having been collected on the filter is compressed to eliminate the fluid clinging to it. The appalling strength of tetanus toxin may readily be appreciated when it is stated that it is twenty times as poisonous as dried cobra venom.

The quantity of the toxin produced in nutrient media varies according to the age of the culture, the composition of the culture fluid, reaction, completeness of the exclusion of oxygen, etc. For some reason more toxin develops in broth inoculated with masses of tetanus spores in nutrient agar, than with bacilli. The variation in strength is partly due to the extreme sensitiveness of the toxin, which deteriorates on keeping at blood heat or on exposure to light. It is sensibly affected by most chemical reagents and is largely destroyed by heating to 55° to 60° C. for a few minutes. It retains its strength best when protected from heat, light, oxygen, and moisture.

The tetanus cultures retain their ability to produce toxins unaltered when kept under suitable conditions; but when subjected to deleterious influences they may entirely lose it.

**Production of Toxin for Immunization of Horses.**—Following is the method of handling stock cultures and of producing toxin, modified by Wilcox in our laboratory from the method used by Anderson and Leake.

**Stock Cultures.**—The stock cultures are grown on a semisolid agar medium neutralized to phenolphthalein.

To transfer cultures, one of the semisolid agar cultures is melted and 1 c.c. is added to a freshly melted semisolid agar tube, at least ten subcultures being thus made from one stock culture. After inoculation, the tubes are cooled, the plugs immersed in paraffin and the tubes incubated at 37° C. After one week's incubation the cultures are stored in the ice-chest, where they may be kept for six months without affecting their ability to produce toxin.

**Preliminary Cultivation.**—Fill potato tubes with about 40 c.c. of the toxin broth (p. 110) sterilize for one and one-half hours on the first day and one hour on the second day. These tubes may be kept for two weeks, up to which time they will give satisfactory growth. To make the first transfer for the preliminary cultivation add 8 c.c. of the melted semisolid agar stock culture of the *B. tetani* to two tubes of glucose broth from which the air has been previously expelled by heating in the Arnold for fifteen to twenty minutes, and which have been cooled down to about 50° C. These tubes are incubated for twenty-four hours and the next day, two freshly heated tubes of broth are inoculated

with 5 c.c. of the glucose broth cultures planted the previous day. On the third day determine the number of flasks that are to be inoculated and inoculate as many freshly heated glucose broth tubes from the second glucose broth generation as there are flasks. Anderson calls for at least six or seven generations in the glucose broth before the inoculation of the toxin broth, but at the Research Laboratory it has been found that three generations, or even two if need be, are sufficient for obtaining a toxicity of 1 to 25,000.

*Inoculation of Toxin Broth.*—The flasks of toxin broth after the second sterilization in the Arnold are ready for inoculation. The broth may be cooled down to 55° to 60° C. by allowing the flasks to stand at room temperature, or in a more rapid way by placing the hot flasks in a large sink, to which cool, and then cold, water is added until the lower portions of the flasks are covered. When the bottoms of the flasks are cool to the hand, the portions above the water being still very hot, the inoculation may be made as follows:

The plugs are carefully removed, the necks flamed and the plugs replaced. In a similar way, the mouths of the culture tubes are sterilized and then, partly removing the plug of a flask, the contents of a potato tube is poured rapidly into a flask. If one prefers, the broth culture may be transferred by using a pipette, but the former method has been used here without subsequent contamination and found very satisfactory. After inoculation, the flasks are incubated for fifteen days at 36° to 37° C., care being taken to exclude all light from them.

The flasks at the end of twenty-four to forty-eight hours show a diffuse cloudiness with the formation of gas bubbles on the surface of the broth. Toward the end of two weeks the gas bubbles usually disappear, while the cloudiness persists and a light precipitate forms at the bottom of the flasks. If it is not convenient to filter on the fifteenth day, the cultures may be kept in the incubator until the twentieth day without a loss of toxicity, but from the twentieth day to the twenty-fifth day the toxin loses about 20 per cent. in potency.

*Filtration.*—All glassware, filters, etc., should be neutral to phenolphthalein and the greatest care should be taken to exclude light, either direct or indirect, by darkening the room and by covering the filtering apparatus with dark cloths, ordinary black cambric being used.

The broth cultures are first passed through Buchner filters about 8 inches in diameter, which have been packed with a layer of finely shredded paper pulp 0.25 inch in thickness. It is of importance that the pulp should be so well packed that the filtrate is absolutely clear, otherwise it will clog the Berkefeld filter. The first filtrate, about 200 c.c., which passes through the Buchner is discarded, as it contains a considerable amount of water from the pulp, and then the filtering of the toxin may proceed. If, after passing 8 to 14 liters through the pulp, the filtrate begins to appear cloudy, the pulp must be discarded and the Buchner repacked. The clear filtrate is then passed through a sterile Berkefeld filter, and 10 per cent. of a 5 per cent. solution of carbolic acid solution is added to the toxin which is now placed in the ice-chest, ready for testing its potency.

*Potency Test.*—Two 350-gram guinea-pigs are inoculated subcutaneously over the abdomen with 1 c.c. of a dilution of 1 to 15,000 and 1 to 25,000 of the toxin respectively. If the toxin has a potency of 25,000 the pig receiving the 15,000 dilution will die on the second to third day and the pig receiving the 25,000 dilution should die on the fourth day. If both pigs die with symptoms of tetanus before the fourth day, the toxin is stronger than 25,000 and a higher dilution should be tested.

**Action of Tetanus Toxin in the Body.**—After the absorption of the poison there is a lapse of time before any effects are noticed. In experimental animals with an enormous amount, such as 90,000 fatal doses, this is about nine hours; with 30,000, ten; with 3000, twelve; with 300, twenty hours; with ten fatal doses, thirty-six to forty-eight hours; with two fatal doses, two or three days; with one fatal dose, four to seven days. Less than a fatal dose will produce local symptoms. The parts first

to be affected with tetanus in these animals are usually the muscles lying in the vicinity of the inoculation—for instance, the hindfoot of a mouse inoculated on that leg is first affected, then the tail, the other foot, the back and chest muscles on both sides, and the forelegs, until finally there is a general tetanus of the entire body. In mild cases, or when a dose too small to be fatal has been received, the tetanic spasm may remain confined to the muscles adjacent to the point of inoculation or infection. The symptoms following a fatal dose of toxin vary greatly with the method of injection. Intra-peritoneal injection is followed by symptoms which can hardly be distinguished from those due to many other poisons. Injection into the brain is followed by restlessness and epileptiform convulsions. In man the first symptoms are usually those of a contraction of the muscles of the lower jaw and then those of the neck. In about one-third of the human cases, the muscles about the site of inoculation are first affected.

**Presence of Tetanus Toxin in the Blood.**—The blood during the first four days of the disease usually contains toxin. After that time antitoxin usually develops and soon makes the blood antitoxic. In St. Louis some years ago the serum of a horse dying of tetanus was given by accident in doses of 5 to 10 c.c. to a number of children, with the development of fatal tetanus. In this connection Bolton and Fisch showed by a series of experiments that much toxin might accumulate in the serum before symptoms became marked.

**Tetanus Antitoxin.**—Behring and Kitasato were the first to show the protective and curative effects of the blood serum of immunized animals. It was found that animals could be protected from tetanus infection by the previous or simultaneous injection of tetanus antitoxin, provided that such antitoxic serum was obtained from a thoroughly immunized animal. From this it was assumed that the same result could be produced in natural tetanus in man. Unfortunately, however, the conditions in the natural disease are very much less favorable, inasmuch as treatment is usually commenced not shortly after the infection has taken place, but some hours after the appearance of tetanic symptoms, when the poison has already attacked the cells of the central nervous system.

**The Production of Tetanus Antitoxin for Therapeutic Purposes.**—The tetanus antitoxin is developed in the same manner as the diphtheria antitoxin—by inoculating the tetanus toxin in increasing doses into horses. The horses receive 5 c.c. as the initial dose of a toxin of which 1 c.c. kills 250,000 grams of guinea-pig in 4 to 6 days and along with this twice the amount of antitoxin required to neutralize it. In five days this dose is doubled. This overneutralized toxin stimulates the production of antitoxin. Recently we have preferred to inject the horses with 5000 units of tetanus antitoxin and then give increasing doses of straight toxin. After some months of this treatment the blood of the horse contains the antitoxin in sufficient amount for therapeutic use. Some horses have produced as high as 600 units per cubic centimeter.

**Antitoxin Unit and Technic of Testing Antitoxin Serum.**—Tetanus antitoxin is tested exactly in the same manner as diphtheria antitoxin, except that the unit is different. In April, 1907, the producers of serum in the United

States agreed to a unit of antitoxin which is approximately ten times the size of the unit of diphtheria antitoxin. A unit is defined as the amount of antitoxin required to just neutralize 1000 minimal fatal doses of tetanus toxin for a 350-gram guinea-pig. The United States government has adopted this unit and supplies the different producers with standardized toxin.

The amount of antitoxic serum which neutralizes an amount of test toxin which would destroy 40,000,000 grams of mouse contains 1 unit of antitoxin by the German standard. In the French method the amount of antitoxin which is required to protect a mouse from a dose of toxin sufficient to kill in four days is determined, and the strength of the antitoxin is stated by determining the amount of serum required to protect 1 gram of animal. If 0.001 c.c. protected a 10-gram mouse the strength of that serum would be 1 to 10,000. The toxin used for testing is preserved by precipitating it with saturated ammonium sulphate and drying and preserving the precipitate in sealed tubes. As required, it is dissolved in 10 per cent. salt solution. For small testing stations the best way is to obtain some freshly standardized antitoxin and compare serums with this.

**Therapeutic Application of Tetanus Antitoxin.**—See Part III.

**Persistence of Antitoxin in the Blood.**—Ransom has clearly shown that the tetanus antitoxin, whether directly injected or whether produced in the body, is eliminated equally rapidly from the blood of an animal, provided that the serum was from an animal of the same species. If from a different species it is much more quickly eliminated.

The same author found some interesting facts in testing the antitoxic values of the serum of an immunized mare, of its foal, and of the milk. The foal's serum was one-third the strength of the mare's and one hundred and fifty times that of the mare's milk. In two months the mare's serum lost two-thirds in antitoxic strength, the foal's five-sixths, and the milk one-half.

**Theories as to the Methods by which the Toxin Produces its Effects.**—Gamprecht and Stintzing concluded from their experiments that the toxin from the wound passed to the central nervous system partly directly by the peri- and endoneural lymph spaces of the nerves which directly connected with subdural space and partly indirectly from the blood. The local tetanus they considered as due to the contact of the poison with the motor end-plates. The experiments of Meyer and Ransom and of Marie and Morax have proved to them that the poison is transported to the central nervous system by the way of the motor nerves—and by no other channel. These authors thought that they had shown that the essential element for the absorption and transportation of the toxin is not the lymph channels, but the axis-cylinder, the intramuscular endings of which the toxin penetrates. The poison is taken up quite rapidly. Marie and Morax were able to demonstrate the poison in the corresponding nerve trunk (sciatic) one and a half hours after the injection. Absorption, however, and conduction are dependent to a large extent on the nerves being intact. A nerve cut across takes very much longer to take up the poison (about twenty-four hours), and a degenerated nerve takes up no poison whatever. In other words, we see that section of the nerve prevents the absorption of the poison by way of the nerve channels. Similarly section of the spinal cord prevents the poison from ascending to the brain. The poison

which passed through the general lymph channels to the blood was partly returned to the tissue fluids throughout the body and taken up by nerve endings and thus produced general tetanus.

According to Meyer and Ransom, the reason why the sensory nerves do not play any role in the conduction of the poison lies in the presence of the spinal ganglion, which places a bar to the advance of the poison. Injections of toxin into the posterior root lead to a tetanus dolorosus, which is characterized by strictly localized sensitiveness to pain.

Ascending centripetally along the motor paths, the poison reaches the motor spinal ganglia on the side of inoculation; then it affects the ganglia of the opposite side, making them hypersensitive. The visible result of this is the highly increased muscle tonus—*i. e.*, rigidity. If the supply continues, the toxin next affects the nearest sensory apparatus; there is an increase in the reflexes, but only when the affected portion is irritated. In the further course of the poisoning the toxin as it ascends continues to affect more and more motor centres, and also the neighboring sensory apparatus. This leads to spasm of all the striated muscles and general reflex tetanus. Zupink showed that local tetanus did not develop when toxin was injected where it did not come in contact with muscle. He believed, therefore, that the muscle spasm was due to direct action of the toxin on the muscle fibers.

Field in our laboratory has shown that not only tetanus toxin, but diphtheria toxin and inert colloids can be demonstrated in the sciatic nerves after they have been injected subcutaneously or intramuscularly, and after varying periods may be found in the spinal cord. He believes that the toxins are absorbed by way of the lymphatics of the nerves, and not by way of the axis-cylinder.

A later experiment of Cernovodeanu and Henni tends to confirm this contention. They ligated all the muscles and bloodvessels in a guinea-pig's leg, leaving intact only the sciatic nerve, skin and bone, and then injected a large amount of tetanus toxin below the point of ligation. The animals in which this was done never developed tetanus. In this case there was only a very slight flow of lymph into the ligated area, and so there could be only a slight flow up the nerve.

If the toxin gets into the blood the only path of absorption to the central nervous system is still by way of the motor-nerve tracts. There seems to be no other direct path, as, for example, by means of the bloodvessels supplying the central nervous system. Even after introducing the poison into the subarachnoid space, owing to the passage of the poison into the blood, there is a general poisoning and not a cerebral tetanus. This at least is the case if care has been taken during the operation to avoid injuring the brain mechanically. A very much smaller amount of toxin is required to produce fatal tetanus if it is injected into a nerve than if it is injected into the blood.

**The Union of Toxin with the Gray Matter of the Brain and Spinal Cord.**—This union is a loose one and the toxin can be partially freed from its union by action of proteolytic ferments. A number of different elements of the cell substances seem to have this power of binding the

toxins. Heating to 65° C. for ten minutes destroys the capacity to fix toxin. These brain substances which unite with toxin are certainly not of the nature of antitoxin, and the brain cells if they produce antitoxin at all certainly share the power with other cells that have no power to bind toxin. Marie, in a recent communication, notes that adrenalin neutralizes tetanus toxin and that lecithin compounds are undoubtedly concerned in the mechanism of tetanus toxin in nerve cells.

**Rapidity of Absorption and Loss of Tetanus Antitoxin from Tissues.—**

The absorption of antitoxin administered subcutaneously takes place rather slowly. In his animal experiments Knorr found the maximum quantity in the blood only after twenty-four to forty-eight hours. From that time on the amount again steadily decreased, so that by the sixth day only one-third the optimum quantity was present. By the twelfth day only one-fiftieth and at the end of three weeks no antitoxin whatever could be demonstrated. We injected one of our laboratory assistants with 10,000 units of tetanus antitoxin subcutaneously. The blood antitoxic strength was found to be as follows: 18 hours, 0.6 unit; 24 hours, 0.8; 48 hours, 1; 72 hours, 1; 148 hours, 0.7. The two important facts to be noted are the slow absorption of antitoxin from the subcutaneous tissues and its long retention in the blood. The two charts showing the absorption and disappearance of diphtheria antitoxin apply equally to tetanus antitoxin (see Figs. 202 and 203, Part III).

When injected intravenously the antitoxin very quickly passes into the lymph. Ransom, in 1901, was able to demonstrate it in the thoracic duct of a dog fifteen minutes after intravenous injection. Only after very massive intravenous doses and a considerable interval of time are small traces found in the cerebrospinal fluid. This is the reason that passively and actively immunized animals become tetanic if the poison is injected directly into the central nervous system or into a peripheral nerve. Antitoxin injected subdurally passes almost entirely over into the blood within twenty-four hours.

So long as the toxin circulates in the blood it is neutralized by antitoxin in about the same proportion as in test-tube experiments. By means of intravenous injections of antitoxin Ransom was able to render the lymph free from toxin in a very few minutes. According to Marie and Morax, toxin injected into the muscles is already demonstrable in the nerve tissue at the end of one and a half hours—*i. e.*, it has already entered the channel, where it is reached with difficulty by the antitoxin. Donitz injected various rabbits intravenously, each with 1 c.c. of a toxin solution containing twelve fatal doses. Thereupon he determined the dose of antitoxin which, when intravenously given, would neutralize this poison after various intervals of time. The antitoxin was of such a strength that in test-tube experiments 1 c.c. of a 1 to 2000 solution just neutralized the amount of toxin employed. He found that at the end of two minutes double the dose required *in vitro* would still neutralize the poison; at the end of four minutes about four times the dose was required, and at the end of eight minutes ten times. When one hour had been allowed to elapse

forty times the original dose just sufficed to protect the animal from death, but not from sickness.

**Differential Diagnosis between Tetanus and Tetanus-like Bacilli.**—The differential diagnosis of the bacillus of tetanus is, generally speaking, not difficult, inasmuch as animal inoculation affords a sure test of the specific organism. No other microorganism known produces effects similar to the tetanus bacillus, nor is any other neutralized by tetanus antitoxin. The other characteristics also of this bacillus are usually distinctive, though microscopic examination alone cannot be depended on to make a differential diagnosis. Difficulty arises when other anaërobic, or aërobic bacilli, almost morphologically identical with the tetanus bacillus, are encountered which are non-pathogenic, such as the *Bacillus pseudotetanicus* (anaërobicus), and the *Bacillus pseudotetanicus* (aërobicus). It is possible, however, that both these bacilli, when characteristic in cultures, are only varieties of the tetanus bacillus, which, under unfavorable conditions of growth, have lost their virulence. These non-virulent types do not, as a rule, have spores absolutely at their ends, and the spores themselves are usually more ovoid than those in the true tetanus bacilli.

**Methods of Examination in a Case of Tetanus.**—(a) *Microscopic.*—From every wound or point of suppuration film preparations should be made and stained with the usual dyes. The typical spore-bearing forms are looked for, but are usually not found. At the same time other bacteria are noted if present.

(b) *Cultures.*—Bits of tissue, pus, cartridge wads, etc., are collected and dropped into glucose bouillon preferably in fermentation tubes with a piece of tissue, etc. (see Isolation).

(c) *Inoculation.*—A salt solution emulsion of material from the wound is inoculated into mice or guinea-pigs subcutaneously. Successful results are usually obtained, if the bacilli or their spores are present.

### **BACILLUS (ANTHRACIS) SYMPTOMATICI (BACILLUS OF SYMPTOMATIC ANTHRAX).**

The bacillus of symptomatic anthrax is the cause of the disease in animals—principally cattle and sheep—known as “black leg,” “quarter evil,” or symptomatic anthrax (rauschbrand, German; charbon symptomatique, French), a disease which is characterized by a peculiar emphysematous swelling of the subcutaneous tissues and muscles, especially over the quarters. Clinically it is sometimes confused with anthrax. Like *B. anthrax*, it is an inhabitant of the soil and is a large spore-bearing organism, but it grows only under anaërobic conditions and is otherwise distinctly different from *B. anthrax*.

**Morphology.**—Bacilli having rounded ends, from  $0.5\mu$  to  $0.6\mu$  broad and from  $3\mu$  to  $5\mu$  long, mostly isolated, also occurring in pairs, joined end-to-end, but never growing out into long filaments, as the anthrax bacilli in culture and the bacilli of malignant edema in the bodies of animals are frequently seen to do. The bacilli are actively motile.

The spores are elliptic in shape, usually thicker than the bacilli, lying near the middle of the rods. This gives to the bacilli containing spores a somewhat spindle shape. It stains readily with the ordinary dyes and is Gram-amphiphile.

**Biology.**—It is a strict anaërobe, developing at room temperature, better at 37° C. It grows best in albuminous or tissue media and when glucose is present. It ferments this sugar with the production of gas. Gelatin is liquefied.

**Growth on Agar.**—The colonies on agar are somewhat more compact than those of malignant edema, but also send out filamentous projections.

**Pathogenicity.**—The bacillus of symptomatic anthrax is pathogenic for cattle, sheep, goats, guinea-pigs, and mice. The guinea-pig is the most susceptible of test animals, and cultures from the heart blood of inoculated guinea-pigs offers the best method of obtaining pure cultures. When susceptible animals are inoculated subcutaneously with pure cultures of this organism, or with spores attached to a silk thread, or with bits of tissue from the affected parts of another animal dead of the disease, death ensues in from twenty-four to thirty-six hours. At the autopsy a bloody serum is found in the subcutaneous tissues, extending from the point of inoculation over the entire surface of the abdomen, and the muscles present a dark red or black appearance, even more intense in color than in malignant edema, and there is a considerable development of gas. The lymphatic glands are markedly hyperemic.

The disease occurs chiefly in cattle, more rarely in sheep and goats; horses are not attacked spontaneously—*i. e.*, by accidental infection. In man infection has never occurred. The usual mode of natural infection by symptomatic anthrax is through wounds which penetrate not only the skin, but the deep, intercellular tissues; some cases of infection by ingestion have been observed. The pathological findings present the conditions above described as occurring in the experimental animals.

**Distribution Outside of the Body.**—Symptomatic anthrax, like anthrax and malignant edema, is a disease due to soil infection, being confined especially to places over which infected herds of cattle have been pastured. By contamination of deep wounds acquired by animals in infected pastures, the disease is spread. The spores are extremely resistant.

**Toxins.**—Under favorable conditions extracellular toxins are formed. Injections of the toxin into animals excite the production of antitoxins.



FIG. 155.—Bacilli of symptomatic anthrax, showing spores. (After Zettnow.)



**Preventive Inoculations.**—Recovery from one attack of symptomatic anthrax protects an animal against a second infection. Active immunity can be produced by vaccines of attenuated organisms. A dried powder of the muscles of animals which have succumbed to the disease is used as a vaccine and subjected to a suitable temperature to insure attenuation of the virulence of the spores contained therein. Two vaccines are prepared—a stronger vaccine by exposing a portion of the powder to a temperature of 85° to 90° C. for six hours, and a weaker vaccine by exposing it for the same time to a temperature of 100° to 104° C. Inoculations are made with this attenuated virus into the end of the tail—first the weaker and later the stronger. The results obtained from this method of preventive inoculation seem to have been very satisfactory.

### THE MALIGNANT EDEMA BACILLUS.

Bacilli of this group are widely distributed, being found in the superficial layers of the soil, in putrefying substances, in foul water. This bacillus was discovered (1877) by Pasteur in animals after infection with putrid flesh, and named by him “vibron septique.” He did not obtain it in pure culture. Koch and Gaffky (1881) carefully studied this microorganism, described it in detail, and gave it the name “*Bacillus edematis maligni*” (Fig. 156).



FIG. 156.—Bacilli of malignant edema. 1, bacilli; 2, with spores; 3 and 4, deep colonies in dextrose nutrient agar. (Kolle and Wassermann.)

**Morphology.**—The edema bacillus is a rod of from 0.8 $\mu$  to 1 $\mu$  in width, and of very varying length, from 2 $\mu$  to 10 $\mu$  or more, according to the conditions of its cultivation and growth. It is usually found in pairs, joined end-to-end, but may occur in chains or long filaments. It is motile, and does not produce a capsule. It forms spores which are situated in or near the middle of the body of the rods, exceptionally near the ends. The spores vary in length and are oval in form, being often of greater diameter than the bacilli, to which they give a more or less oval shape.

The bacilli stain readily by the usual aniline colors employed and are Gram-amphophile.

**Biology.**—An obligate anaërobe growing best on or in albuminous media but also growing well on ordinary media especially if available carbohydrates are present. Growth occurs at 20° C., but is more rapid and abundant at 37° C.

**Growth on Agar.**—On dextrose agar plates the colonies appear as dull, whitish points, irregular in outline, and when examined under a low-power lens are seen to be composed of a dense network of interlacing threads, radiating irregularly from the centre toward the periphery.

**Growth in Gelatin.**—The colonies are similar to those on agar, a liquefied zone developing after several days.

**Resistance.**—The spores are very resistant and because of this the soil remains infected.

**Other Media.**—Milk is coagulated and then digested. Blood serum is liquefied. An offensive odor develops due to the proteolysis. Glucose is fermented with the production of acid and gas.

**Pathogenicity.**—Malignant edema is mostly confined to domestic animals, horse, sheep, cattle and swine. It follows the contamination of wounds with infected soil or other infectious material; and also occurs as a complication of surgical operations. It is therefore a frequent contaminant after war wounds. The depth of the wound as well as the introduction of foreign particles and other bacteria are factors in infection, as the inoculation of washed spores frequently fail to produce infection. An extensive hemorrhagic edema of the subcutaneous tissues develops from the site of the wound. The serous effusion is frothy from gas production and has a foul odor. As a rule in the larger animals the bacilli do not invade the blood until after death. The bacillus is pathogenic for the smaller laboratory animals, rabbit, guinea-pig, mouse; a septicemia developing as well as the local edema.

**Immunity.**—Recovery from infection is followed by immunity. It is claimed that an extracellular toxin is produced, also a leukocidin.

#### **BACILLUS WELCHII GROUP (BACILLUS AËROGENES CAPSULATUS).**

The first bacillus of this group to be described minutely was found by Welch in the bloodvessels of a patient suffering with aortic aneurysm; at autopsy, made in cool weather, eight hours after death, the vessels were observed to be full of gas bubbles. Since then it has been found in many cases in which gas has developed from within sixty hours of death until some hours after death. It occurs most frequently after external cutting operations and wounds. These cases are, as a rule, marked by delirium, rapid pulse, high temperature, and the development of emphysema and discoloration of the diseased area or of marked abdominal distention when the peritoneal cavity is involved. Members of this group are present, as a rule, in the intestinal canal of man and animals and are apt to be found in the dust of hospitals and elsewhere. Herter has shown that they are present in excessive numbers in certain diseases of the digestive tract. These cases are apt to develop anemia.

Different strains of bacilli belonging to this group have appeared under different names and their exact relationship is still uncertain. Thus, *B. phlegmonis emphysematosæ* of Fränkel is probably the same as *B. welchii*. *B. perfringens* (Veillon and Zuber, 1898) and *B. enteritidis sporogenes* (Klein, 1895) are closely related if not the same organism.

**Morphology.**—Straight or slightly curved rods, with rounded or sometimes square-cut ends; somewhat thicker than the anthrax bacilli and varying in length; occasionally long threads and chains are seen. The bacilli in the animal body, and sometimes in cultures, are enclosed in a transparent capsule. Spores are usually absent in the tissues and often in cultures. Dunham showed that the culture isolated by Welch formed spores when grown on blood serum. Some strains since isolated make spores readily.

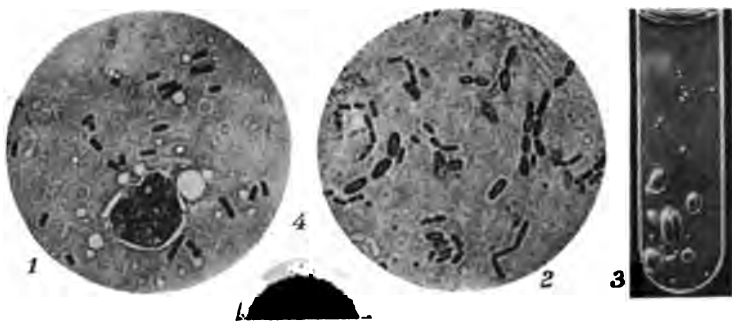


FIG. 157.—*Bacillus aerogenes capsulatus*. 1, bacilli; 2, spores; 3, culture in dextrose-nutrient agar.

**Biology.**—Anaerobic, non-motile, non-liquefying bacilli. They are positive to Gram, but are more easily decolorized than many bacteria. Growth is rapid at 37° C., in the usual culture media in the absence of oxygen, and is accompanied by the production of gas. *Nutrient gelatin* is not liquefied, but it is gradually peptonized. On *agar* colonies are developed which are from 1 to 2 mm. or more in diameter, grayish-white in color, and in the form of flattened spheres, ovals, or irregular masses, covered with hair-like projections. *Bouillon* is diffusely clouded, and a white sediment is formed. *Milk* becomes acidified and coagulated, then partially digested, giving a worm-eaten appearance to the clot. A large amount of butyric acid is produced.

**Isolation.**—When quantities up to 2.5 c.c. of fresh bouillon cultures are injected into the circulation of rabbits and the animals killed shortly after the injection, the bacilli develop rapidly, with an abundant formation of gas in the bloodvessels and organs, especially the liver. This procedure is one of the best methods of obtaining the bacilli. The material suspected of containing the bacillus alone or associated with other bacteria is injected intravenously into rabbits, which are killed five minutes later and kept at 37° C. for sixteen hours, and cultures made from the liver and heart blood.

**Pathogenicity.**—Its pathogenicity is usually not marked in healthy animals, although Dunham found that the bacillus taken freshly from human infection is sometimes very virulent.

As we have said bacilli of this type are one of the frequent infections after irregular unclean wounds such as those received in war. In these infections there is marked destruction of tissue, especially in the muscles. This is due possibly to the large quantity of butyric acid produced from the glycogen of the muscles. Many attempts have been made to demonstrate a specific toxin production by these bacilli (Harde, Weinberg) but the results have not been clear-cut. Robertson has made a study of the isolation and types of anaërobes found in wounds.

It is suggested by Welch that in some of the cases in which death has been attributed to the entrance of air into the veins the gas found at the autopsy may not have been atmospheric air, but may have been produced by this or some similar microorganism entering the circulation and developing shortly before and after death. The same may be true for gas in the uterine cavity.

### BACILLUS BOTULINUS.

This bacillus, while not pathogenic for man, is as first shown by Van Ermengen the causative agent in a characteristic type of food poisoning, the symptoms being due to the toxin produced by the growth of this bacillus in foods. The clinical symptoms of this type of food poisoning are neuromuscular in character. There are secretory disturbances as increase or suppression of the salivary secretions or a thick secretion of mucus in the mouth and pharynx. Disturbances of accommodation, ptosis, double vision, and dysphagia are the common motor symptoms. Obstinate constipation and retention of urine as well as disturbances in heart action and respiration occur. Fever is absent. Death is not uncommon and is due to respiratory paralysis.

**Morphology.**—The bacilli are large, 4 to 6 $\mu$  long and 0.9 to 1.2 $\mu$  wide, with somewhat rounded ends. They are slightly motile, Gram-positive, and have oval spores, usually terminal. Short chains may be produced.

**Biology.**—It is anaërobic, growing best at 22° to 25° C. Gelatin is liquefied and glucose fermented with the production of acid and gas. A butyric acid odor develops in cultures. The colonies on gelatin are yellowish, translucent, coarsely granular, the granules moving slowly when liquefaction begins. The older colonies are brownish, more opaque and show fine thorn-like extensions.

**Resistance.**—The spores are not very resistant to heat, being killed in fifteen minutes at 85° C. or in one-half hour at 80° C.

**Toxin Production.**—A thermolabile extracellular toxin is produced in glucose-broth cultures. Guinea-pigs, rabbits, mice, cats and monkeys are susceptible to the toxin and succumb not only to injection but also when the toxin is given by mouth. As little as 0.0001 c.c. may produce symptoms. After an incubation period, dyspnea, convulsions and

paralysis occur and death is due to respiratory paralysis. The paralyzes are due to degeneration of the ganglion cells of the anterior horn and of the bulbar centres. According to Dickson this is secondary to disturbances in blood supply due to thrombosis associated with meningeal hemorrhage. An antitoxin has been produced.

**Foods Involved.**—Meat and meat preparations, such as canned or pickled meats and sausages are the usual offending foods. Until recently it was thought that preserved or canned fruits or vegetable products were not a factor. Cases due to such foods have been reported and Dickson has shown that the toxin develops in a medium of peas or beans. All these foods have one thing in common, they are prepared for weeks or months before they are consumed. The source of contamination is not definitely known, though the organism has been isolated from pigs' feces.

**Diagnosis of Botulism.**—The diagnosis usually rests on the clinical manifestations. Verification may be attempted by examination of the suspected food. A thick emulsion of the food is made in saline and from it glucose gelatin plates and fermentation tubes (with tissue) are inoculated. A portion of the suspension can be heated (60° C., one-half hour) and similarly inoculated. From the growth in the fermentation tube, heated and unheated anaërobic plates are made. The pure cultures obtained are then tested for toxin production by animal inoculation. Saline extracts of the suspected food, if they produce characteristic symptoms in experimental animals, confirm the clinical diagnosis in man.

**Prophylaxis.**—Although a rancid butter odor may be present in contaminated foods, it may be slight and not recognized. Cooking will destroy the toxin. Cleanliness in preparation is an aid in preventing contamination and also in limiting the numbers of associated bacteria which, by their growth, would aid in establishing anaërobic conditions. Brine for pickling should contain at least 10 per cent. of salt as this concentration prevents the growth of the bacilli. Incomplete sterilization is the source of danger with canned goods.

#### **BACILLUS FUSIFORMIS (BACILLUS OF VINCENT'S ANGINA).**

This organism together with spirochetes was found by Plaut and Vincent in pseudomembranous inflammation of the throat (Vincent's Angina). The constancy of its presence and the preponderance of their numbers in smears from this condition suggests strongly their etiological connection with the disease. Fusiform bacilli and spirochetes are also encountered in gangrene, noma, ulcerative stomatitis, gingivitis, dental caries and even around the gum margins of dirty teeth, especially if there are deposits of tartar. Although *B. fusiformis* is present in many conditions, either because of the greater virulence of certain strains or because of reduced resistance of the tissues they, together with the spirochetes are probably the essential agent in the production of Vincent's angina and noma and act as a contributing factor in other lesions.

The fusiform bacilli are anaërobic and have been cultivated. The most

successful methods of isolation and cultivation are those of Krumwiede and Pratt who studied fifteen strains from various conditions. The typical bacillus is double-pointed, containing one or more granules. In culture the morphology is variable. In fluid media there is a tendency to produce filamentous types, which may form tangled, thread-like masses. The colonies are characterized by thread-like outgrowths. The fifteen cultures studied fell into saccharose fermenting and saccharose non-fermenting groups, but this difference had no correlation to the source of the culture. The fusiform bacillus and the spirochetes accompanying it in the lesion were never encountered in cultures made from single colonies although Tunncliffe claimed that the fusiform bacillus and the spirochete were only different forms of the same organism.



FIG. 158.—Vincent's bacillus with accompanying spirochetes.

### BACILLUS TYPHI-EXANTHEMATICI.

**Historical.**—The bacillus typhi-exanthematici was first isolated by Plotz, in 1914, from the blood of individuals suffering from typhus fever. A year later, Plotz, Olitsky and Baehr published the report of an extensive study of bacteriological, serological and animal investigations which contained much evidence that this organism is the causative agent in typhus fever. Continued investigations by these workers and by others working in coöperation with them have made the evidence stronger. The following description is from their reports and from a private communication from Dr. Baehr, giving information of work to be reported in the *Journal of Infectious Diseases* early in 1917.

**Morphology.**—The organism is a small, slender bacillus, the average length being about one micron. In young cultures the organisms are small and uniform in size; in very old subcultures there is often an admixture of various sized bacilli from coccoid forms up to some measuring two microns in length. The bacilli are usually straight, though slightly curved forms occur occasionally. The ends are slightly pointed, less often rounded. (Fig. 159.) With special stains an occasional organism will show a fine polar body at one end, more rarely at both.

**Reaction to the Gram Stain.**—The organism in subcultures is Gram-positive. According to Olitsky, Denzer and Husk and Baehr and Plotz, the colonies in occasional blood cultures may consist only of exceedingly minute bacilli which are completely decolorized by Gram's method. In subsequent subcultures these bacilli always become Gram-positive. A similar experience was the rule in organisms isolated from typhus infected lice.



FIG. 159.—Typhus bacillus. (Plotz.)

**Cultural Characteristics.**—The bacillus typhi-exanthematici is an obligate anaërobe. After artificial cultivation for many months, it has been possible to obtain slight aërobic growth with occasional strains.

The organism, as far as tested, grows only upon a medium containing ascitic or hydrocele fluid and glucose. Although growth occurs in fluid media containing these ingredients, the optimum medium contains 1 part of ascitic fluid and 2 parts of 2 per cent. glucose agar. Even on slants of this medium a large amount of culture material must be subinoculated in order to obtain growth and it is advisable to smear it in thick streaks upon the surface. The subcultures upon such slants incubated in Buchner tubes containing equal parts of pyrogalllic acid and 30 per cent. sodium hydrate solution usually show evidences of growth after about three days. The growth is profuse by the sixth or seventh day and at that time is soft and creamy in character, raised above the surface and of a glistening white color. In the medium itself a diffuse clouding gradually develops as the growth increases (the precipitation phenomenon of Libman).

Strains of the *Bacillus typhi-exanthematici* isolated in the United States, Mexico, Serbia and Russia are identical in their action upon sugars. They invariably ferment glucose, maltose, galactose and inulin with marked acid production and precipitation of protein, but have no action upon saccharose, lactose, raffinose, arabinose, dextrin and mannit.

**Method of Isolating the Organism from the Blood.**—The method used in making the anaërobic blood culture is that of Liborius-Veillon, using the ascitic fluid glucose agar recommended by Libman as the optimum medium for bacterial cultivation.

The essential ingredient of the medium is ascitic or hydrocele fluid. It must be clear, free of bile or blood pigment and should possess a specific gravity of more than 1015. Filtered ascitic fluid or one which contains a preservative or has been sterilized by heating should not be used. Pleural fluids are probably useless.

The technic of the culture consists in withdrawing with a syringe and

needle 15 c.c. of blood from a vein and dividing this among 8 large test-tubes (20 x 2 cm.) half-filled with melted 2 per cent. glucose agar. To each tube in turn, ascitic or hydrocele fluid equivalent to at least one-third of the volume of the agar (*i. e.*, 6 to 10 c.c.) is added. The contents are then poured once or twice into another sterile test-tube in order to secure thorough mixing. After thorough solidification of the mixture, each tube is covered with a layer of plain agar, 2 or 3 cm. deep, and then the cotton stopper paraffined. The tubes are then incubated at 37° C.

**Appearance of Colonies.**—Colonies usually appear in the culture tubes in about nine or ten days. Occasionally they may occur as early as the fifth or as late as the nineteenth day. They first appear as small opaque spots which, by direct light, are white. During the subsequent two or three days they grow rapidly larger, usually assuming a "Y" shape, and develop a brownish zone of precipitation in the medium about them. In consistency the growth is always exceedingly soft, and the color, especially of the older colonies, is pale brown.

The organism may be isolated from the blood during the entire febrile course of the disease, from the first to the last day. In two cases Plotz was still able to grow the bacillus from the blood twelve and thirty-six hours respectively after the crisis. In the mild cases only one to three colonies may develop in the eight tubes; in severe cases as many as ten to twenty may be present. During chills which occur frequently at the onset of the illness and occasionally during its first week numerous colonies develop from the blood indicating that enormous numbers of the bacteria may be present in the blood.

The percentage of positive blood cultures also varies with the severity of the disease, as can be seen from the following table taken from an article by Baehr and Plotz:

Type of disease	Mortality per cent.	Number of cases studied.	Percentage with positive blood cultures per cent.
New York typhus (Brill's disease) . . . . .	0.2	34	53
Russian typhus . . . . .	5.5	24	79
Mexican typhus . . . . .	20.0	8	100
Balkan typhus, 1914 . . . . .	18 to 60	7	100

The organisms can also be pretty regularly isolated from monkeys and guinea-pigs in which the disease has been produced by the inoculation of typhus blood.

**Pathogenicity.**—On artificial media the organism loses its pathogenicity very rapidly. Organisms which have been grown on artificial media for less than a week are sometimes still pathogenic for guinea-pigs but those cultivated for longer periods are always non-pathogenic. After an incubation period more irregular than of typhus fever, the animals may develop a febrile illness during which in several cases the bacillus has been recovered from the blood.

**Development of Antibodies after Typhus Fever.**—Specific agglutinins, precipitins, opsonins and complement-fixing antibodies are regularly present in the blood of typhus convalescents. They usually appear during the second week of the disease, increase as the crisis is approached, reach their maximal titer during the first or second week of convalescence and usually persist in the blood for months, in one case for two and one-half years. The curve of the course of development of the antibodies in this disease is typically an immunity curve.



Baehr has made the observation that individuals who have been intimately exposed to typhus infection, may develop antibodies in their blood without having fever or other clinical evidences of the disease. None of these people developed the disease, although subsequently they were repeatedly exposed to infection. Some adults who are not known to have had typhus fever have been shown to have immune bodies in their blood.

**Prophylactic Immunization.**—The repeated subcutaneous inoculation in human beings of vaccine made from these organisms is followed by the development in their blood of specific antibodies. In guinea-pigs, however, although they develop immunity after an infection due to the blood, such an antibody production does not occur and thus far it has also been found impossible to confer immunity upon these animals by vaccination. Plotz, Olitsky and Baehr have recently carried out investigations in the Balkans and in Volhynia, Russia, where typhus fever was epidemic, upon the value of prophylactic immunization in human beings, and have reported that vaccination with *Bacillus typhi-exanthematici* markedly reduces the incidence of the disease.

**Transmission by the Body Louse.**—The stomach of typhus infected lice contains immense numbers of bacilli which are morphologically identical with the *Bacillus typhi-exanthematici*. Such bacilli have been seen in typhus lice in Mexico by Ricketts and Wilder and Olitsky, Denzer and Husk. In smear preparations made from typhus lice, these bacillary organisms are decolorizable by Gram's method. Olitsky, Denzer and Husk succeeded in cultivating them from the lice by the anaerobic method described above, and found that after several generations on artificial media the bacilli gradually became Gram-positive. The bacilli were then morphologically, culturally and serologically identical with the *Bacillus typhi-exanthematici* and in the early subcultures were pathogenic for guinea-pigs.

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## CHAPTER XXXIV.

### THE CHOLERA SPIRILLUM (CHOLERA VIBRIO) AND SIMILAR VARIETIES.

IN 1883 Koch separated a characteristically curved organism from the dejecta and intestines of cholera patients—the so-called “*comma bacillus*.” It was absent from the blood and viscera, and was found only in the intestines; and the greater the number, it was said, the more acute the attack. Koch also demonstrated an invasion of the mucosa and its glands. The organisms were found in the stools on staining the mucous flakes or the fluid with methylene blue or fuchsin, and sometimes alone; by means of cultivation on gelatin they were readily separated from the stools. Numerous control observations made upon other diarrheic dejecta and upon normal stools were negative; the comma bacillus was found in choleraic material only, or occasionally in small numbers in the stools of healthy persons who came in contact with cholera. Soon, however, other observers described comma-shaped organisms of non-choleraic origin. Finkler and Prior, for instance, found them in the diarrheal stools of cholera nostras, Deneke in cheese, Lewis and Miller in saliva. All of these organisms, however, differed in some respects from Koch’s comma bacillus, and it has since been proved that none of them is affected by the specific serum of animals immunized to cholera. After a time, therefore, the exclusive association of Koch’s vibrio with cholera or those in contact with it became almost generally acknowledged, until now it is regarded by bacteriologists everywhere to be the specific cause of Asiatic cholera. Certain sporadic cases of cholera-like disease, however, are undoubtedly due to other organisms.

**Morphology.**—Curved rods with rounded ends which do not lie in the same plane, of an average of  $1\frac{1}{2}\mu$  in length and about  $0.4\mu$  in breadth. The curvature of the rods may be very slight, like that of a comma, or distinctly marked, particularly in fresh unstained preparations where the adhesion of two individuals presents the appearance of a half-circle. By the inverse junction of two vibrios S-shaped forms are produced. Longer forms are rarely seen in the intestinal discharges or from the cultures grown on solid media, but in fluids, especially when grown under unfavorable conditions, long, spiral filaments may develop. The spiral forms are best studied in the hanging drop, for in the dried and stained preparations the spiral character of the long filaments is often obliterated. In film preparations from the intestinal contents in typical cases it will be found that the organisms are present in enormous numbers, and often in almost pure culture. In old cultures

irregularly clubbed and thickened involution forms are frequent, and the presence in the organisms of small, rounded, highly refractile bodies is often noted.

**Staining.**—The cholera spirillum *stains* with the aniline colors usually employed, but not as readily as many other bacteria; a diluted aqueous solution of carbol-fuchsin (1 to 10) is the most reliable staining agent. It is decolorized by Gram's method. The organisms exhibit one long, fine flagellum attached to one end (other spirilla often have two or more end flagella).

**Biology.**—The cholera spirillum is *aërobic*, *liquefying*, and *extremely motile*. It grows readily on ordinary culture media, best at 37° C., but also at room temperature, 22° C.

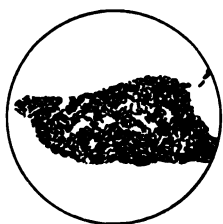


FIG. 160.—Contact smear of colony of cholera spirilla from agar.  $\times 700$  diameters. (Dunham.)



FIG. 161.—Cholera spirilla preparation from gelatin-plate culture of cholera.  $\times 800$  diameters.

**Gelatin Plate Cultures.**—In gelatin plate cultures, characteristic colonies are produced. After twenty-four hours' growth, there is a uniform granular appearance of the surface, which because of the high degree of refraction gives the appearance of being powdered with broken glass. Color is practically absent or there may be a slight yellowish tint thus differing from *B. coli*. If growth is continued, liquefaction appears about the colony and its appearance gradually changes. The characteristic colony in gelatin used to be of the greatest practical importance. With the introduction of special selective media, however, the use of gelatin has been practically discontinued.

**Gelatin Stab Culture.**—In gelatin stab culture, a small funnel of liquefaction appears after twenty-four hours. This deepens and broadens, until at the end of a week liquefaction may be complete.

**Agar.**—On agar a moist, shiny, grayish-yellow layer develops. On the surface of alkaline-agar plates the individual colonies are characteristic. They are round, transparent and have a rather distinctive opalescent sheen. This characteristic appearance is made use of for isolation.

On **Dieudonné alkaline-blood agar** the colonies are large and circular. On transmitted light there is a glassy transparency, on reflected light they are grayish. On **alkaline-egg agar** the colonies, when examined

by transmitted light, appear to be deep in the agar and have a distinctive hazy appearance due to the development of a halo about the colony. A zone of clearing may develop where growth is continued. As some fecal bacteria other than vibrios may develop on these media, the development of a typical colony is a great aid in isolation.

**Löffler's blood serum** is rapidly liquefied at 37° C. **Milk** is not coagulated. **Glucose** and **saccharose** are fermented with great rapidity, acid only being formed. Growth in fluid media is abundant and usually characteristic, most so in peptone-water. **Peptone-water** is diffusely cloudy to a moderate degree, but at the surface the cloud is much denser. Due to the greater supply of oxygen, the vibrios seek the surface and multiply there more freely.

**Reaction of Media.**—Cholera grows best on media that are strongly alkaline to litmus. They can grow in an excess of alkali sufficient to inhibit the growth of many of the bacteria found in feces. This is most evident where an alkali-albumin mixture is used to increase the alkalinity. (See Special Media.)

**Cholera-red Reaction.**—All cholera strains give this reaction. This is important as many non-cholera spirilla do not give this reaction.

**Changes due to Artificial Cultivation.**—Cholera strains which have been in cultivation for some time show more spiral forms. They grow less typically or not at all on selective media and fluid cultures may develop a wrinkled pellicle. Their digestive powers are also lessened, as gelatin-liquefaction or the liquefaction of Löffler's serum medium. Variations can also be induced by special conditions.

**Hemolysin Production.**—Six strains were isolated by Gottshlich at El Tor from Pilgrims to Mecca, who died with diarrheal symptoms but had shown no clinical evidences of cholera. These strains are called "El Tor" strains. They give the serum reactions of cholera vibrios but produce a strong hemolysin. Kraus and Ruffer believed them not to be true cholera, as hemolysin production was considered by the former as an attribute possessed only by non-cholera types. Further investigations have shown that cholera vibrios may develop, lose, or show wide variations in their ability to produce hemolysins. It is apparently a potential power of all vibrios and of no value in differentiation. For this reason the El Tor strains must be considered as cholera vibrios.

**Resistance and Vitality.**—**Development Outside of Body.**—If a culture is spread on a cover-glass and exposed to the action of the air at room temperature the spirilla will be dead at the end of two or three hours, unless the layer of culture is very thick, in which case it may take twenty-four hours or more to kill all the spirilla. This indicates that



FIG. 162.—Cholera colonies in gelatin; twenty-four to thirty-six hours' growth.  $\times$  about 20 diameters.

infection is rarely if ever produced by means of dust or other dried objects contaminated with cholera spirilla. The transmission of these organisms through the air, therefore, can only take place for short distances, as by the spray from infectious liquids.

The cholera bacillus is also injuriously affected by the abundant growth of saprophytic bacteria. It is true that when associated with other bacteria, if present in large numbers, and if the conditions for their development are particularly favorable, the comma bacillus may at first gain the upper hand, as in the moist linen of cholera patients, or in soil impregnated with cholera dejecta; but later, after two or three days, even in such cases, the bacilli die off and other bacteria gradually take their place. Thus, Koch found that the fluid contents of privies twenty-four hours after the introduction of comma bacilli no longer contained the living organisms; in impure river water they were not demonstrable for more than six or seven days, as a rule. In the dejecta of cholera patients they were found usually only for a few days (one to three days), though rarely they have been observed for twenty to thirty days, and on one occasion for one hundred and twenty days. In unsterilized water they may also retain their vitality for a relatively long time; thus, in stagnant well water they have been found for eighteen days, and in an aquarium containing plants and fishes, the water of which was inoculated with cholera germs, they were isolated several months later from the mud at the bottom. Koch found them in the foul water of a tank in India, used by the natives for drinking purposes. In running river water, however, they have not been observed for over six to eight days. In milk they are finally destroyed by acidity due to the growth of the milk bacteria, in sterilized milk they may survive eight to ten days. For the cholera organisms the conditions favorable to growth are a warm temperature, moisture, a good supply of oxygen, and a considerable proportion of organic material. These conditions are fully met, outside the body, in very few localities.

The comma bacillus has the average resistance of spore-free bacteria, and is killed by exposure to moist heat at 56° C. in half an hour, at 80° in five minutes, at 95° to 100° C. in one minute. The bacilli have been found alive kept after a few days in ice, but ice which has been preserved for several weeks does not contain living bacilli.

Chemical disinfectants readily destroy the vitality of cholera vibrios. For disinfection on a small scale, as for washing the hands when contaminated with cholera infection, a 0.1 per cent. solution of bichloride of mercury, or a 2 to 3 per cent. solution of carbolic acid or, better, lysol may be used. For disinfection on a large scale, as for the disinfection of cholera stools, strongly alkaline milk of lime is an excellent agent. The wash of cholera patients, contaminated furniture, floors, etc., may be disinfected by a solution of 5 per cent. carbolic acid and soap water. For the disinfection of drinking water, chlorinated lime, 1 or 2 parts per million of free chlorine may be used for fairly pure water. Five parts per million would probably be effective even in polluted water.

**Pathogenesis.**—Not one of the lower animals is naturally subject to cholera. Koch succeeded in producing symptoms and intestinal lesions in guinea-pigs similar to those in man by introducing cultures by catheter after neutralizing the contents of the stomach with a solution of carbonate of soda and inhibiting peristalsis by the use of opium. Thomas injected a dilute suspension of cholera spirilla into the ear vein of young rabbits, and thus caused symptoms and lesions of the intestinal mucous membrane resembling those of cholera in man. The spirillum was recovered from the feces. Metchnikoff was successful with suckling rabbits by rubbing a small amount of a culture on the teats of a mother rabbit. Intraperitoneal injections with cholera spirilla kill guinea-pigs acutely, but intestinal lesions are rarely found.

**Accidental Human Infection.**—There are several cases on record which furnish the most satisfactory evidence that the cholera spirillum is able to produce the disease in man. In 1884 a student in Koch's laboratory in Berlin, who was taking a course on cholera, became ill with a severe attack of cholera. At that time there was no cholera in Germany, and the infection could not have been produced in any other way than through the cholera cultures which were being used for the instruction of students. In 1892 Pettenkofer and Emmerich experimented on themselves by swallowing small quantities of fresh cholera cultures obtained from Hamburg. Pettenkofer was affected with a mild attack of cholera or severe diarrhea, from which he recovered in a few days without any serious effects, but Emmerich became very ill. On the night following the infection he was attacked by frequent evacuations of the characteristic rice-water type, cramps, tympanites, and great prostration. His voice became hoarse, and the secretion of urine was somewhat diminished; this condition lasting for several days. In both cases the cholera spirillum was obtained in pure culture from the dejecta. Finally, there is the case of Dr. Oergel, of Hamburg, who accidentally, while experimenting on a guinea-pig, allowed some of the infected peritoneal fluid to squirt into his mouth. He was taken ill and died a few days afterward of typical cholera, though at the time of his death there was no cholera in the city.

**Lesions in Man.**—Cholera in man is an infective process of the epithelium of the intestine, in which the spirilla clinging to and between the epithelial cells produce a partial or entire necrosis and final destruction of the epithelial covering, which thus renders possible the absorption of the cholera toxin formed by the growth of the spirilla. The larger the surface of the mucous membrane infected and the more luxuriant the development of bacilli and the production of toxin, the more pronounced will be the poisoning, ending fatally in a toxic paralysis of the circulatory and thermic centres. On the other hand, however, there may be cases where, in spite of the large number of cholera bacilli present in the dejecta, severe symptoms of intoxication may be absent. In such cases the destruction of epithelium is not produced or is so slight that the toxic substance absorbed is not in sufficient concentration to give rise to the algid stage of the disease, or for some reason the spirilla do not produce toxin to any extent.

**Distribution in the Body.**—The cholera spirilla are found only in the intestines and are believed never to be present in the blood or

internal organs. The lower half of the small intestine is most affected, a large part of its surface epithelium becoming shed. The flakes floating in the rice-water discharges consist mostly of masses of epithelial cells and mucus, among which are numerous spirilla. The spirilla also penetrate the follicles of Lieberkühn, and may be seen lying between the basement membrane and the epithelial lining, which become loosened by their action. They are rarely found in the connective tissue beneath, and never penetrate deeply. In more chronic cases other microorganisms play a greater part and deeper lesions of the intestines may occur.

**Cholera Toxins.**—Koch assumed that the severe symptoms of cholera were due to the absorption of a toxin produced by the growth of the vibrio in the intestines. The toxic effects are apparently due to substances which are an integral part of the organism and are only liberated by the breaking down of the vibrio. Suspensions of killed vibrios, when injected into animals, give the same symptoms as living cultures, although quantitatively less toxic. The endotoxin is labile, and is best shown in cultures which are killed by chloroform or by heating to 56° C. for one hour. More active chemicals or a higher degree of heat changes it from a more specific toxin to a more general protein poison. The bacteria-free filtrates of fresh fluid cultures are only slightly toxic; old cultures, however, due to breaking down of the vibrios may be very toxic. This toxicity is due mostly to substances similar in action to the general class of ptomaines. Kraus has, however, been able to demonstrate, what he considers an extracellular toxin, in young broth cultures. Metchnikoff and Roux have also attempted to prove the existence of an extracellular toxin by growing cholera vibrios in collodion sacs implanted in the peritoneum of guinea-pigs. The production of an antitoxin against such toxins has not been accomplished.

**Communicability.—Origin of Epidemics.**—The two fundamental epidemiological facts are, that the vibrio leaves the body only in the feces, and the mode of infection is by way of the mouth. The feces of the *cholera patient* during the acute stage of the disease is extremely rich in vibrios, which are at times present in almost pure culture. As the case recovers they decrease in number, but persist after recovery for seven to fourteen days, in exceptional instances longer. In only one case have they persisted for three months, that is, chronic carriers do not exist. These persons constitute the "*convalescent carriers*." In this connection, the mild cases which are undiagnosed or overlooked are important. Another group of persons may act as sources of infection, viz., excrete cholera spirilla in their stools. These are the "*healthy or contact carriers*." Not all persons who ingest cholera develop the disease. In a number, the vibrio will multiply in the intestine to a limited extent and be excreted in the stools, although no clinical evidences of disease are present. These healthy carriers are important not only as insidious spreaders of infection, but they may be potential cases of cholera. Should their resistance be lowered they may develop the disease.

The transfer of the infectious agent to the mouth may occur in

several ways: by personal contact, by fomites, and by contamination of food and water. Where a moderate number of cases are developing in a district having fair sanitary conditions, contact, especially with mild cases and carriers, or indirectly, fomites or infection of food are the sources of infection. As other factors enter, such as the contamination of the soil and privy-vaults with subsequent infection of well and river water and of green vegetables, the cases increase in number. Where a general water supply becomes contaminated an explosive, widespread epidemic follows. It is easy to understand how localized epidemics are kept active, by the development of healthy carriers, who help maintain the contamination of their surroundings; and, given also the climatic conditions, such as heat and moisture, to favor the continued vitality or even multiplication outside of the body, how endemic foci persist. The transfer of infection by fomites, such as body and bed linen or dishes, etc., is only dangerous when direct. Drying quickly lessens the danger. Flies may be a factor by mechanical transfer of the virus.

The susceptibility to infection of different individuals varies and conditions may lower or raise the resistance of the individual. The occurrence of healthy carriers illustrates this. Such carriers may be very numerous. Abel and Clausen, for example, found that 14 of 17 persons belonging to families of 7 cholera patients, had cholera vibrios in their stools. In a group of immigrants who were exposed, we found 10 per cent. were healthy carriers.

The resistance of the individual depends upon his general good health. Gastric and intestinal disorders due to indiscreet eating or drinking, or other causes undoubtedly favor infection or in the case of healthy carriers may cause the development of cholera. At the New York Quarantine Station two such cases promptly developed: one after the administration of a dose of salts, the other after a drinking bout.

**Cholera Immunity.**—Eight to ten days after recovery the serum of cholera patients contains protective substances. If a guinea-pig is injected intraperitoneally with living cholera vibrios, and serum from the patient be given, the pig recovers. Similar protective substances are found in the serum of animals injected with sublethal doses of live vibrios or with killed organisms. The serum is not antitoxic, for although it will protect an animal from a lethal dose of living vibrios, by preventing their multiplication, it has little effect, when a fatal dose of killed organisms or toxic extracts is given. Similarly an animal may be highly immune to the injection of living cultures but on intestinal infection will show no resistance to the poisonous products absorbed.

The antibodies present in the serum are precipitins, agglutinins, opsonins, bacteriolytic and bactericidal substances.

**Prophylactic Vaccination and Serum Therapy.**—See Part III.

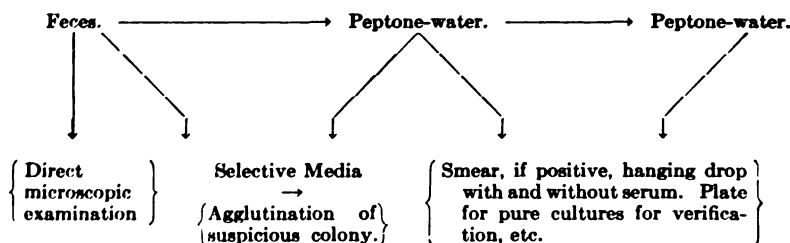
**Specific Serum Reactions.**—**Agglutinins.**—Because of the acuteness of the disease, the agglutination reaction is valueless for diagnosis. It may be of diagnostic help in recovered cases, where no bacteriological



diagnosis has been made. Agglutination is used, however, for identification of the cholera vibrio. In this way cholera and non-cholera vibrios can be separated with certainty, because a cholera-immune serum usually contains few group agglutinins for non-cholera types. Freshly isolated strains agglutinate freely; in fact, seem to give better reactions than stock cultures, although strains vary in their agglutinability; relatively inagglutinable strains such as are encountered among freshly isolated strains of typhoid are not found.

**Pfeiffer Phenomenon.**—This phenomenon (see page 178), which is a bactericidal test *in vivo*, can be employed to differentiate the cholera vibrio from other vibrios. The bactericidal serum should have a high titer, viz., 1 c.c. of a 1 to 1000 dilution should be able to dissolve a loop of cholera vibrios when injected intraperitoneally together. Each factor in the test must be controlled.

### ISOLATION OF CHOLERA VIBRIO FROM STOOLS. OUTLINE OF PROCEDURE.



**Direct Microscopic Examination.**—This is of great value in suspected cases but of no use in the examination of carriers. The presence of many typical, extremely motile vibrios warrants a tentative diagnosis of cholera. In exceptional cases, not cholera, a great abundance of vibrios may be found. This led us in one instance unnecessarily to isolate a nurse, who while caring for a cholera patient, developed nervous diarrhea.

**Peptone-water.**—Inoculate with feces and incubate six to twelve hours. Examine smears from surface growth. If the vibrios are numerous, prepare hanging drops with and without immune serum. If vibrios are not found in the smear, or if too few in number for hanging drop observation, subinoculate into peptone-water, or selective media, or both.

**Subculture Peptone-water.**—This second enrichment is probably never required except in the examination of suspected carriers. In four instances we have found cholera vibrios in subculture where they were not evident in smears from the first peptone culture. This second enrichment helps also to exclude some of the vibrios other than cholera; some will have died out, some will not have enriched beyond the amount present in the first peptone culture; these are not cholera. Smears from the surface growth are examined and if positive, examination

of hanging drops carried on. Where haste is not a factor as in carrier examinations the examination of the first peptone culture may be omitted.

In making smears and hanging drops from peptone-water especial care should be taken not to shake the tubes and that the loop is just touched to the surface. In the examination of smears little time need be spent; if very few vibrios are present, further enrichment is necessary. If they are present in sufficient numbers for agglutination, they are found at once.

The use of the surface growth for agglutination is open to certain criticisms, but in practical work it has given us accurate results. Where cholera or cholera-like vibrios, having the same ability to enrich as true cholera, are present, the surface growth is nearly a pure culture. The reliability of the peptone method, as outlined, was best shown in a series of examinations where we found two carriers. In this series 50 per cent. of the stools contained vibrios. The results were checked by the isolation of pure cultures. With some experience a great number of examinations can be carried through in this way, using peptone-water only, with a minimum of preparation and equipment.

**Selective Media.**—Inoculation may be done directly from the feces or after enrichment in peptone-water. The advantage of such media is that they may be heavily inoculated. The colonies which develop are used for agglutination either in hanging drop, or more convenient, the macroscopic slide method (see under Typhoid). Various modifications of Dieudonné's have been suggested. The alkaline-egg medium has the advantage that a distinctive vibrio colony develops allowing quick selection for agglutination should non-vibrios develop. A secondary plating on plain agar is necessary, if pure cultures are to be obtained from selective media. The other bacteria, which are only restrained, may be transferred in fishing a colony and thus yield mixed cultures.

Alkaline agar may be employed for plating either directly from the feces or from peptone enrichments. As stated, the colony is distinctive and no difficulty will be encountered if the vibrios are fairly numerous.<sup>1</sup>

**Saccharose Peptone-water.**—This medium has been suggested by Bendick to avoid the time-consuming microscopic examination of peptone tubes. The stool is first inoculated in peptone-water and the surface growth then inoculated into this medium. Because of the ability of the cholera vibrio to rapidly split saccharose, decolorization occurs in five to eight hours. The tubes which do not decolorize in this time can be discarded, those decolorized are examined for vibrios, which, if present, are isolated by plating.

Because of the presence of a fermentable sugar, the growth is diffused and the surface is not satisfactory for agglutination. To avoid this difficulty duplicate peptone tubes could be planted, however, and used

<sup>1</sup> The direct plating of stools on agar or gelatin is sometimes of practical importance. In no disease but cholera do vibrio colonies approach or exceed in number those developing from the ordinary fecal bacteria. Even without identification by agglutination, such a condition gives us a practically certain diagnosis of cholera.

for agglutination when the saccharose peptone tubes were decolorized. This method promises to be of the greatest value where many specimens must be examined in the search for carriers.

**Examination of Suspected Carriers.**—The simplest procedures possible must be employed when the daily examinations may run into many hundreds or even thousands. The peptone method outlined reduces the bacteriological work very much, especially if the first peptone tubes are not examined. The collection of stools under these circumstances is impracticable. Ordinary swabs moistened in peptone-water may be inserted into the rectum to obtain fecal material. Individual glass specula may be employed to aid in the introduction of the swabs. Where feasible much time is saved by giving an identification card a number and dropping the swab at the time of taking into a similarly numbered tube of peptone-water. The sterilization of the peptone tubes, supported in blocks of wood or racks protected by a cover instead of by cotton plugs, and the use of individual wire loops (sterilized in bundles) for transfers or smears saves a great deal of time. The removal and replacing of cotton plugs and the burning of the platinum loop usually employed is thus avoided. In transferring, the wire is dropped into the second tube. The exposure of the tubes when the cover is removed for inoculation or transfer does not lead to interference by contamination.

**Isolation from Water.**—As the cholera vibrios are few in number in water a large volume should be used. About 1 liter of water is taken and 100 c.c. amounts placed in Ehrlenmeyer flasks and to each is added 10 c.c. of a tenfold strength peptone-water. These are shaken and incubated for eighteen hours. The surface growth is then subinoculated in peptone-water for further enrichment if necessary and plates made for isolation. Especial care must be taken in fishing as vibrios other than cholera may be present in the water, and there is some evidence that cholera vibrios lose their agglutinability to some extent after living in water. These difficulties may be surmounted by many fishings and the use of a high titre serum in low dilutions in order to select cultures for final identification.

**Spirilla (Vibrios) More or Less Allied to the Cholera Spirillum.**—Various types of spirilla may be isolated from stools, water, and other sources. Some are practically identical, morphologically and culturally, with true cholera. It would be well to limit the term "cholera-like vibrios" to this group. Another group of vibrios, similar to cholera in some respects but differing in others might be termed "non-cholera vibrios." Much of the practical interest in these types was lost when the serological methods for identification were introduced. In emergency work, where immune serum is not at hand, they are of extreme practical importance. A few of the types are of interest because of their pathogenicity for animals.

Some of these vibrios enrich in mixed cultures in peptone-water like true cholera, others enrich to a limited extent or die out. The majority of the vibrios found in stools during routine examinations for cholera spirilla can be excluded culturally if serum be not available. Of 50 vibrios thus isolated we found that 43 did not give the cholera-red reaction. Of the 7 that did, 2 produced no acid from glucose and 4

produced acid and gas. One produced acid only, but could be excluded, as it formed a tenacious pellicle on peptone-water.

The following is a short list of vibrios of interest historically or because of their pathogenicity for animals:

*Vibrio metschnikovii*; source, epizootic gastro-enteritis of fowls, present in intestinal contents and in blood. Typical vibrio with one flagellum, liquefies gelatin and gives cholera-red reaction. A minute amount of culture inoculated into a cutaneous wound causes a fatal vibriosepticemia in pigeons and guinea-pigs.

*Vibrio massawah*; source, stools, considered cholera vibrio when first isolated, four flagella, pathogenicity like *Vibrio metschnikovii*.

*Vibrio septicus*; source, stools, case of cholera, cholera-like culturally and morphologically, minute amounts cause a rapidly fatal septicemia in guinea-pigs.

*Spirillum finkler-prior*; source, feces in cholera nostras, does not give cholera-red reaction.

*Vibrio ivanoff* and *Vibrio berlionensis*; source, former artificially inoculated stools for disinfection tests, latter, water artificially inoculated to determine viability of cholera vibrio in water. Both are probably variants produced by artificial conditions; they give the immune reactions of cholera spirilla.

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## CHAPTER XXXV.

### PATHOGENIC MICROÖRGANISMS BELONGING TO THE HIGHER BACTERIA (TRICHOMYCETES).

OBSERVERS are still of different opinions in regard to the classification of this group of organisms (see Chapter II, p. 41).

Foulerton and his associates have made an extensive study of this group, both saprophytic and parasitic varieties, and they agree with some others in calling attention to the acid-fast character of some of the varieties and to the apparent relationship of the group to *B. tuberculosis*, *B. mallei*, and *B. diptheriae*. To us, however, the relationship does not seem to be close enough to place all of these organisms in one group. We have shown (Chapter II) that the apparent branching in *B. diptheriae* is not a true branching. If these are not classed with the true bacteria, they should either be put in a group by themselves or be classed with the cladothrix group since their apparent branching takes place in a manner similar to that described as occurring in the latter group.

Foulerton considers all organisms in the group classed as higher bacteria as belonging to a single genus, streptothrix, which he places with the hyphomycetes, or mold fungi, because of their growth in branching threads from spore-like bodies. He says that streptothrix and actinomyces are absolutely synonymous terms, and that the majority of pathologists consider them so.

Wright and others do not agree with this view (see below). More minute work, both clinical and experimental, should be done on this group of infections before a classification can be accepted.

**Leptothrix Infections.**—Leptothrix forms are frequently found in the human mouth (*Leptothrix buccalis*), and a few writers have claimed that under certain conditions these may become pathogenic, but since no corroborative work has been done, and very little is known about the group, no opinion can be formed of the worth of these observations.

**Cladothrix Infections.**—The organisms found in the comparatively few cases which have been considered by their observers to be due to cladothrix have not been minutely enough studied to decide definitely as to their true or false branching, the characteristic chosen to separate them from the nocardia; hence it is difficult to separate the two groups, but an attempt should be made, since the difference said to exist between them is an important one from a morphological stand-point. Clinically, however, according to the reports, the cases cited are very similar to those said to be due to nocardia (streptothrix) and to actinomyces.

*Gastén* found in a case of clinically typical actinomycosis, in which abscess cavities were found along the spinal column, not the usual actinomyces in the yellow, granular pus, but a fine mass of filaments. Cultures grew on all the ordinary media, best at incubator temperature, but also at lower temperature on gelatin. The gelatin-stick culture, which was especially characteristic, formed on the surface

a whitish button; delicate threads stretched out in all directions from the point of inoculation. On agar and potato rumpled, folded films formed on the surface, with white deposit which contained spores. Animal inoculation gave positive results only in a few cases of intra-peritoneal injection of rabbits and guinea-pigs. Purulent nodules were found in the peritoneum. Gasten called the organism *Cladothrix liquefaciens*.

*Eppinger* found on postmortem examination of a case of chronic cerebral abscess, which was the result of purulent meningitis, in the pus and abscess walls, etc., a delicate fungoid growth which he succeeded in cultivating on various media. On sugar agar it formed yellow, rumpled colonies which finally developed into a skin. On potato it grew rapidly, but the colonies remained small, at first a white granular deposit, which afterward turned red, and on the twentieth day resembled a crystallized almond. It did not grow well on gelatin. In bouillon it formed on the surface a small white granule, which became deeper in the centre as it grew and sank to the bottom as a white deposit. The bouillon remained clear.

Microscopically the fungus consisted of fine threads without branches which exhibited distinct motility. No flagella were observed. It was judged to be a cladothrix, to which the name "asteroides" was given by the author. It proved to be quite pathogenic for rabbits and guinea-pigs, and produced an infection called pseudotuberculosis. Mice were not affected by inoculation.

### THE ACTINOMYCES.

The little clumps produced by this group of parasites were first seen by von Langenbeck in 1845, and the organisms were later discovered by Bollinger (1877) in the ox. They were given the name of actinomyces, or ray fungus, by the botanist Harz. They were reported in human beings by Israel in 1878.

The characteristics of the microorganisms, described first by Boström (1890) and then by Wolf and Israel (1891), differed greatly and have led to confusion. Boström's organism grew best aërobically and developed well at room temperature. He noted the intimate relation of the organism to those on fragments of grain, and this led to the finding of similar microorganisms in the outer world on grains, grasses, etc. There is no doubt that some suppurative processes have been due to organisms having these characteristics, but they do not seem to excite true actinomycosis.

Wolf and Israel described a microorganism from two human cases, which differs from that described by Boström, but agrees with the microorganisms obtained by most of the more recent investigators from typical actinomycosis. It grew best under anaërobic conditions and did not grow at room temperature. Its growth was much less luxuriant than Boström's microorganism. On the surface of anaërobic agar slant cultures on the third, fourth, and fifth day numerous minute

isolated dew-drop-like colonies appeared, the largest pin-head in size. These gradually became larger and formed ball-like, irregularly rounded, elevated nodules varying in size up to that of a millet seed, exceptionally attaining the size of a lentil or larger. As a rule the colonies did not become confluent, and an apparently homogeneous layer of growth was seen to be made up of separate nodules if examined with a lens. In some instances the colonies presented a prominent centre with a lobulated margin and appeared as rosettes. A characteristic of the colonies was that they sent into the agar root-like projections. In aërobic agar slant cultures no growth or a slow and very feeble growth was obtained. In stab cultures the growth was sometimes limited to the lower portion of the line of inoculation or was more vigorous there. In bouillon, after three to five days, growth appeared as small white flakes, partly floating and partly collected at the bottom of the

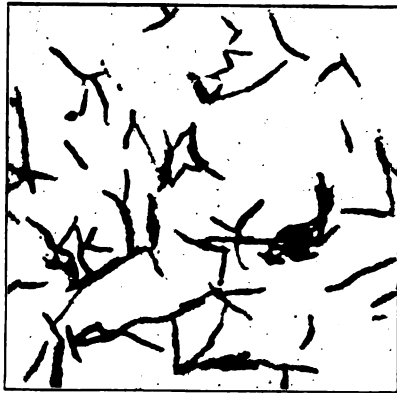


FIG. 163.—Smear from bouillon culture of actinomycetes.  $\times 1500$  diameters.  
(From Wright.)

tube. Growth occurred in bouillon under aërobic conditions, but was better under anaërobic conditions. The organisms grew in branching and interlacing filaments, which later tend to break into segments (see Fig. 162). The microörganism in smear preparations from agar cultures appeared chiefly as short homogeneous, usually straight, but also comma-like or bowed rods, whose length and breadth varied. In many cultures short, plump rods predominated, and in others longer, thicker, or thinner individuals were more numerous. The ends of the rods often showed oval or ball-like swellings. Swollen clubs were formed irregularly in the presence of blood or serous fluids.

On intraperitoneal inoculation guinea-pigs and rabbits after four to seventeen weeks showed, after being killed, tumor growths mostly in the peritoneal cavity and in one instance in the spleen. Microscopic examination of the tumors showed in all cases but one the presence of typical actinomycetes colonies, in most cases with typical "clubs." The

general histological appearance of the tumors was like that of actinomycotic tissue.

Wolf in a later paper reports that an animal inoculated in the peritoneal cavity with a culture of the same organism had lived a year and a half. At the autopsy several tumors were found in the peritoneal cavity, and in the liver a large typical tumor in which were many colonies which by microscopic examination were shown to be typical club-bearing actinomyces colonies.

Wright, in 1905, made an extensive study of actinomycosis and added greatly to our knowledge of it.

**Naked-eye Appearance of Colonies of Parasite in Tissues.**—In both man and animals they can be readily seen in the pus from the affected regions as small, white, yellowish or greenish granules of pin-head size (from 0.5 to 2 mm. in diameter). When pus has not formed they lie embedded in the granulation tissue.

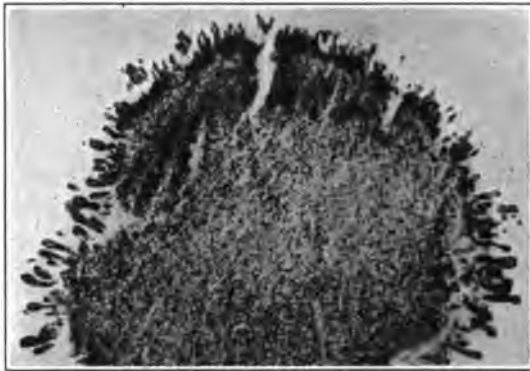


FIG. 164.—A typical "club"-bearing colony of actinomyces.  $\times 325$  diameters. (From Wright.)

**Microscopic Appearance.**—Microscopically these bodies are seen to be made up of threads which radiate from a centre and present bulbous, club-like terminations (Fig. 164). These club-like terminations are characteristic of the actinomyces. They are generally arranged in pairs, closely crowded together, and are very glistening in appearance. They are more common in bovine than in human lesions. They have been thought to be reproductive elements, but they are probably simply a reaction of the filament end to the host tissue. The threads which compose the central mass of the granules are from  $0.3\mu$  to  $0.5\mu$  in diameter. The threads show true branching and in the older colonies show a segmentation which gives them the appearance of chains of cocci. Sometimes the whole centre of the colonies seems to be a mass of coccus-like bodies most of which are considered spores or conidia; the clubs are from  $6\mu$  to  $8\mu$  in diameter.

The threads and spores are *stained* with the ordinary aniline colors, also by Gram's solution; when stained with gentian violet and by Gram's



method the threads appear more distinct than when stained with methylene blue. The clubs usually lose their stain by Gram's method and take the contrast strain.

**Isolation of Actinomyces.**—Certain strains of actinomyces grow aërobically and others anaërobically. The anaërobic strains are grown with difficulty. A large number of solidified blood serum or serum agar tubes are inoculated with the hope that one or two will develop a growth. The cultures appear much like those of tubercle bacilli. They grow, however, into the medium, and take on a yellowish hue. Wright recommends that granules, preferably obtained from closed lesions, are first thoroughly washed in sterile water or bouillon and then crushed between two sterile glass slides. (In bovine cases make sure the granule has filamentous masses, for, if not, no culture will grow.) The crushed granule is transferred to a tube of melted 1 per cent. glucose agar at 40° C. The material is thoroughly distributed by shaking and the tube placed in the incubator. A number of granules after washing should be placed on the inside of a sterile test-tube and allowed to dry. In this way, should the material be contaminated, the drying of the granules for several weeks may kill off the other organisms. The tube should be examined daily. If a number of living filaments were added to the agar a large number of colonies will develop. These will be most numerous in a zone five to twelve millimeters below the surface (microaërophiles).

The cultures are quite resistant to outside influences; dried, they may be kept for a year or more; they are killed by an exposure of five minutes to a temperature of 75° C.

**Experimental Inoculation in Animals.**—True progressive infection is rarely or never obtained by the injection of pure cultures into rabbits, guinea-pigs, or other small animals. In cattle, however, the disease has been produced from cultures. The cultures form the characteristic "club"-bearing colonies in the tissues of experimental animals. These colonies are either enclosed in small nodules of connective tissue or are contained in suppurative foci within nodular tumors made up of connective tissues in varying stages of development.

Wright does not accept the prevalent belief, based on the work of Boström, Gasperini, and others, that the specific infectious agent of actinomycosis is to be found among certain branching microörganisms, widely disseminated in the outer world. He thinks that these forms belong to a separate genus, *Nocardia*, and that those cases of undoubted infection by them should be called nocardiosis and not actinomycosis. The term actinomycosis should be used only for those inflammatory processes the lesions of which contain the characteristic granules or nodules. That *Nocardia* ever forms these characteristic structures in lesions produced by them has not been convincingly shown.

**Occurrence.**—Actinomycosis is quite prevalent among cattle, in which it occurs endemically; it is more rare among swine and horses. Many cases have in recent years been reported in man. The disease is rarely communicated from one animal to another and no case is known where a direct history of human contagion has been obtained. The cereal grains, which from their nature are capable of penetrating

the tissues, have been found in centres of actinomycotic infection in the lower animals. The microörganism may also be introduced by means of carious teeth. Cutaneous infection has been produced by wood splinters, and infection of the lungs by aspiration of fragments of teeth containing the fungus. The presence of the microörganism in cereal grains, which was formerly accepted, is denied by Wright and therefore certainly placed in doubt. The further distribution of the fungus after it is introduced into the tissues is effected partly by its growth and partly by conveyance by means of the lymphatics and leukocytes. Not infrequently a mixed infection with the pyogenic cocci occurs in actinomycosis.

**Characteristics of Disease in Man and Animals.**—In the earliest stages of its growth the parasite gives rise to a small granulation tumor, not unlike that produced by the tubercle bacillus, which contains, in addition to small round cells, epithelial elements and giant cells. After it reaches a certain size there is great proliferation of the surrounding connective tissue, and the growth may, particularly in the jaw, look like, and was long mistaken for, osteosarcoma. Finally, suppuration occurs, which, according to Israel, may be produced directly by the fungus itself.

The course of the disease is very chronic. Usually the first sign is a point of infiltration about the lower jaw or lower on the neck. This almost painless swelling increases and finally softens in its centre. The necrotic tissue finally forces a passage externally or, passing downward, infects the pleura, lungs, mediastinum, or ribs. As a rule the disease is not accompanied by fever. In cattle the disease is usually situated in some portion of the head, especially in the jaw, tongue, or tonsils, hence called lumpy jaw, wooden tongue, etc. Primary lung, intestinal, and skin lesions are not infrequent. These local lesions sometimes scatter and produce a general infection and the udder may be involved.

**Treatment.**—In 1892 Nocard showed that cases in animals might be cured by iodide of potassium, calling attention to the fact that Thomassen had recommended this treatment in 1885. It is given in doses of  $1\frac{1}{2}$  to  $2\frac{1}{2}$  drams once a day. Salmon and Smith (U. S. Bureau of Animal Industry, Circular No. 96) give directions as to its use.

**Mycetoma (Madura Foot).**—This is a purulent inflammation of the foot occurring primarily in warm climates. The inflammation is accompanied by much irregular enlargement of the foot. Three varieties of this condition have been described based upon the color of the granules found in the diseased area: (1) white, (2) black, and (3) red. The white variety has been studied by Musgrave and Clegg (1907), who have isolated an organism resembling somewhat actinomyces and somewhat the organism isolated by Wright (1898) from a black variety of the disease which is probably a true mold. (See "Trench Foot.")

#### NOCARDIA (STREPTOTHRIX) INFECTIONS.

The most familiar name of this group of microörganisms is streptothrix, but this name had already been used for another genus; there-

fore, according to the rules of nomenclature, nocardia, which name was proposed by Trevisan in 1889 for the organism discovered by Nocard in *farcin des boeufs*, should be employed. Wright calls attention to the misuse of the term *streptothrix*, and gives the reasons for the employment of the term *nocardia* in its place.

From widely scattered localities and at long intervals of time reports have been published describing unique cases of disease produced by varieties of microörganisms belonging to the genus nocardia. In some of these cases, points of similarity can be recognized in the clinical symptoms and the gross pathological lesions, while others differ widely in both respects. Nocardia have been found in brain abscess, cerebro-spinal meningitis, pneumonic areas, and in other pathological conditions. Eppinger injected cultures into guinea-pigs and rabbits, and observed that they caused lesions similar to tuberculosis. Consolidation of portions of both lungs, thickening of the peritoneum, and scattered nodules resembling tubercles were noted by Flexner in a case of human infection due to nocardia in which the pathological picture of the disease resembled so nearly that of tuberculosis in human beings that the two diseases could be separated only by finding the causative microörganism in each case. But in no two cases reported up to the present time have the descriptions of the microörganisms found agreed in all particulars. In some cases no attempt at cultivation was made. In other cases numerous and careful plants on various culture media failed to develop the specific organism. As late as the year 1904 Tuttle was able to find the reports of only twelve cases in which nocardia was found in sufficient abundance to have been an important, if not the principal, factor in producing disease. These cases were all fatal, and only once was the character of the disease recognized during life. As the clinical symptoms and the lesions in the human subject as well as in the animals experimentally inoculated with nocardia often resemble those of miliary tuberculosis, the question is naturally suggested whether cases of nocardia tuberculosis are not more numerous than the few reported cases would indicate. The almost universal prevalence of genuine tuberculosis and the extreme gravity of the disease have so long occupied the attention and study of the medical profession that much is taken for granted, and in cases in which the symptoms and lesions resemble with some closeness those characteristic of the well-known disease they may easily be set down without question to the account of the tubercle bacillus. The cases of nocardiosis reported which simulated tuberculosis have been fatal, and the lesions for the most part have been widely distributed, but in a number of cases old lesions have been found which suggest that the disease may have been localized for a longer or shorter time, and then, by some accident, may have become rapidly general. In this respect also these cases may resemble tuberculosis. Whether all cases of nocardiosis in the human subject are general and fatal or, as in tuberculosis and actinomycosis, whether there may be cases of localized disease which recover, are questions which have not yet been decided. The methods employed

to demonstrate the presence of tubercle bacilli render nocardia more or less invisible. Again, unless the observer keeps in mind the possibility of nocardia infection, he may not appreciate the importance of finding slender threads with or without branches, and may consider them accidental bacilli, or varieties of leptothrix or non-pathogenic fungi.

As the lungs have appeared to be the seat of the primary infection in most of the cases of human nocardiosis it is very desirable that all cases presenting the physical signs of tuberculosis, in which repeated examinations fail to discover the tubercle bacillus, should be systematically examined for threads. In this way alone can the frequency of the disease be determined. Gram's method of staining is one of the most reliable agents for demonstrating these organisms. Varieties of nocardia are widely distributed and are not very infrequently met with, but as yet, with the exceptions mentioned above, very little is known about them.

Tuttle's report of a case of general nocardia infection at the Presbyterian Hospital gives such a good clinical, bacteriological, and pathological picture of an acute case of this infection that a considerable portion of it is repeated here:

Six days before her admission to the hospital her illness began with a severe chill and fever and pain in her left side and back. The following day the pain in the side was worse and breathing was difficult. She began to cough and had some expectoration, but no blood was noticed in the sputa. At irregular intervals she had alternating hot and chilly sensations.

On admission the patient complained of pain in the left side of the chest, cough, fever, weakness, and prostration. Her temperature was 103° and her pulse and respirations were rapid.

The history of the disease and the physical signs indicated an attack of acute lobar pneumonia, the area of consolidation being small and situated in the lower part of the left upper lobe in front. Frequent and violent coughing, with almost no expectoration, pain in the affected side and in the lumbar region, restlessness and sleeplessness, and involuntary urination were the symptoms noted during the first four days in the hospital. The pneumonic area increased somewhat and extended backward to the posterior axillary line, and the temperature was continuous at 103° to 103.5°. On the fifth day the temperature fell 2° and signs of resolution appeared in the consolidated area. The apparent improvement, however, was of short duration. On the sixth day the temperature rose to 104.5°, and continued to rise each day, reaching 107.5° shortly before death, which occurred on the ninth day in the hospital and the fifteenth day of the disease. There were repeated attacks of profuse sweating. On the day before her death three indurated swellings beneath the skin were noticed. One, on the left forearm, about the size of a walnut, apparently contained pus. Two, of smaller size, were situated in the right groin.

Blood cultures from a vein in the arm, taken on the sixth day, remained sterile. The leukocyte count on the seventh day was 36,000.

**Autopsy.**—On the right arm, the left forearm, the abdominal wall, and on both thighs there are eight to ten slightly projecting, rounded, fluctuating, subcutaneous swellings from  $\frac{1}{2}$  to 1 inch in diameter. The skin over most of these nodules is unaltered, but over the larger ones there is a slight bluish discoloration. The nodules are composed of bluish gray, thick, mucilaginous matter, which is very tenacious and can be drawn out into long threads. The lower lobe is thickly studded with miliary tubercles, and scattered through the

entire lung are suppurating foci. Liver and spleen normal. Kidneys: The surface is evenly dotted with minute white spots, which suggest septic emboli rather than tubercles. A few prominent white nodules from  $\frac{1}{4}$  to  $\frac{1}{2}$  inch in diameter, contain thick, tenacious matter (Fig. 166). Section shows that the entire substance of the kidney is densely studded with these minute white granules.

The gross pathological conditions were interpreted before nocardia was found as follows: An old tuberculous nodule in the right lung; acute miliary tuberculosis in the right lung and peritoneum; acute lobar pneumonia, affecting the left lung; septic infarctions and pyemic abscesses of both lungs, heart muscle, both kidneys, pancreas, mesenteric lymph nodes, and subcutaneous connective tissue. The miliary tubercles of the right lung and peritoneum presented the characteristic appearance of genuine tuberculosis. They were minute, hard, gray, almost translucent nodules, while the granules in the kidneys were of an opaque white or yellowish-white color.

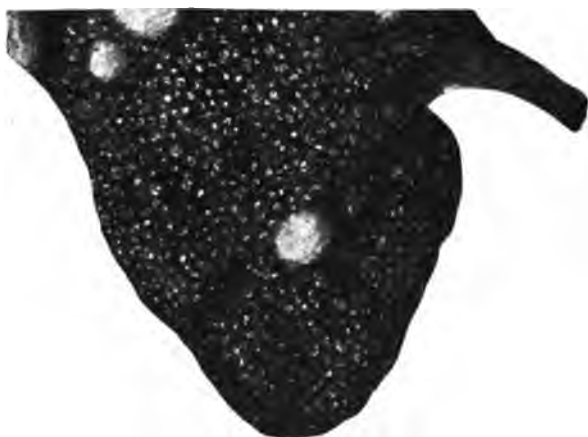


FIG. 165.—Portion of kidney showing minute and large areas of infection.

**Microscopic Examination.**—Smears from the abscess beneath the skin and on the surface of the kidneys were stained with methyl blue, carbol-fuchsin, and by Gram's method. The smears resemble those made of tenacious sputum. There is a large amount of mucoid material containing a considerable number of leukocytes. Occasionally irregularly curved, thread-shaped microorganisms are found. They vary considerably in length and thickness, and broken and apparently degenerating fragments are seen. The more slender threads are evenly stained, but some fragmentation or beading of the protoplasm can generally be observed. The thicker threads and broken fragments show deeply stained globules and irregular bodies in a faintly visible rod or thread-shaped covering. Some branching threads are observed, but more commonly they are not branching. No other microorganisms are found in the smears. Sections from the lower lobe of the right lung, stained with hematoxylin and eosin, show in certain places the identical microscopic appearances which are considered characteristic of tuberculosis. Stained by Gram's method, with care not to decolorize too completely, threads like those described in the abscesses are found

in great abundance, but rather faintly stained. No threads can be found within the typical tubercles with giant cells, but in the zones of small cells around them they are seen in great numbers, winding about among the cells and forming a sort of network. In the minute foci of small cells one or two fragments of threads are generally seen, and a moderate number in the small abscesses. In the areas of more diffuse infiltration these threads are abundant. No other microorganisms can be found except in the pneumonic area of the left lung, where some groups of cocci are seen.



FIG. 166.—*Streptothrix* from bouillon culture. (From Tuttle.)

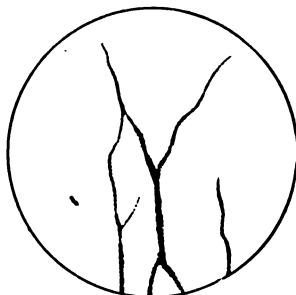


FIG. 167.—Young *streptothrix* threads showing terminal buds. (From Tuttle.)

**Culture Experiments.**—Six tubes of Löffler blood serum were inoculated from the kidneys and kept at 37° C. On the third day minute white colonies appeared in some of the tubes, and on the fifth day all the tubes showed from three to ten or twelve similar colonies in each. The colonies increased in size until some of them reached a diameter of one-eighth of an inch. The color, at first white, changed to yellowish-white and then to a decided pale yellow. The well-developed colonies cling firmly to the surface of the medium and were not easily detached or broken up. The growths in all of the tubes were absolutely pure, and consisted of branching threads like those found in the sections.

Löffler's blood serum seems to be the most suitable medium for cultures. The growth on this medium is more rapid and abundant than on any of the other media tried.

On plain agar and glycerin agar the growth is the same as on blood serum, but is less rapidly developed.

In bouillon the growth is slow. If the tube is not disturbed or jarred, minute white tufts are seen clinging to the surface of the glass. But if the tube is shaken even slightly they sink slowly to the bottom, forming a white, fluffy layer. These growths when undisturbed resemble minute balls of thistle-down. The yellow color is not apparent even in the mass at the bottom of the tube.

It is strictly aerobic.

**Morphology** (Figs. 166 and 167).—On blood serum the threads are comparatively thick and coarse, but those growing in bouillon are very slender and delicate. The main trunk also is often thicker than

the branches. When unstained they are homogeneous gray threads, without any appearance of a central canal or double-contoured wall. There is never any segmentation of the threads. When properly stained there is always a distinct beading or fragmentation of the protoplasm, but overstaining with fuchsin produces rather coarse, evenly stained rods. The branching is irregular and without symmetry, and the branches are placed at a wide angle, very nearly, and sometimes quite, at right angles. This is best seen in specimens taken from liquid media. The irregularly stellate arrangement of the branches, which was observed by Eppinger in his original specimen, is often seen in young organisms floated out from a liquid medium.

**Spore Formation.**—On examining the deep orange or red-colored growth upon potato, one is surprised to find that the threads have entirely disappeared and that the specimen consists of moderately large coccoid forms. These represent the spore form of the organism, and when planted upon blood serum the branching threads again appear. The spores stain readily with carbol-fuchsin and are not easily decolorized. They are spherical, or nearly so, but often appear somewhat elongated, apparently from beginning germination. They are killed by exposure to moist heat of 65° to 70° C. for an hour, but are more resistant to dry heat. Drying destroys the threads after a comparatively short time, but the spores retain their vitality for an indefinite period. A dried-up potato culture retains its vitality at the end of almost four years.

The identity of Tuttle's microorganism is not fully established. It is undoubtedly a nocardia, but it does not agree in all particulars with any of the varieties described.

**Animal Inoculations.**—A number of rabbits and guinea-pigs were inoculated subcutaneously upon the abdomen and in the neighborhood of the cervical, axillary, and inguinal lymph nodes with colonies broken up in salt solution. Indurated swellings were produced at the point of inoculation and a number of abscesses resulted. The abscesses developed rapidly and some of them opened spontaneously, while others were incised. The material evacuated did not resemble ordinary pus, but was thick and mucilaginous and exceedingly tenacious, like that from the subcutaneous abscesses of the patient described above. The microscopic appearance was the same, and the nocardia threads were found in considerable numbers. Several rabbits and guinea-pigs and two cats received peritoneal inoculations, but none of them showed any sign of infection. When rabbits were inoculated intravenously, a rapidly fatal general infection was produced, and the lesions were similar in kind and distribution to those described in the human subject.

**Other Cases Reported.**—*Ferré* and *Faguet* found in Bordeaux, in a cerebral abscess in the centrum ovale, a branching fungus, colored by Gram, which corresponded to nocardia. It grew on agar in round, ochre-colored colonies; on potato there was little growth visible; slimy, tough colonies, which became gray and remained free from white dusting on the surface. Inoculations in rabbits and guinea-pigs were negative.

Varieties of nocardia have been found in the human vagina. We have found a variety of nocardia in several cases of stillbirth with invasion of the placenta with the same organism.

Numerous cases have been observed in which nocardia proved to be the cause of chronic lung diseases, clinically suspected to be tuberculosis.

**Treatment.**—Recently autogenous vaccines have been tried in certain cases, but it is yet too soon to determine with what result.

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## CHAPTER XXXVI.

### FILTRABLE VIRUSES. DISEASES OF UNKNOWN ETIOLOGY.

#### FILTRABLE VIRUSES.

THERE exists a class of infectious diseases from which it has been quite impossible up to the present time to demonstrate visibly any individual microorganisms, although infective material from such diseases may, with certain precautions, be passed through stone filters of varying degrees of porosity (for types of filters, see p. 93); the filtrates will contain the virus and be capable of reproducing the disease with all its characteristics when inoculated into a susceptible animal. Examined microscopically, even with the highest powers, the filtrate is limpid, and, except in a few diseases which will be described in detail later on, not a sign of characteristic particulate matter can be seen. Such a filtrate therefore contains either ultramicroscopic organisms or small organisms with refraction and staining powers so faint that they cannot be demonstrated by our present methods.

Certain precautions must be observed in such filtrations. In the first place the filter must be shown by actual test to be free from infection. Any and all of the test organisms must be absolutely retained and none pass into the filtrate. A few minute species of germs have been found to pass certain grades of filters, especially under pressure. Thus the bacillus of guinea-pig pneumonia, which is  $0.5\mu \times 0.7\mu$ , passes Berkefeld V (Wherry); a spirillum isolated by von Esmarch passes, according to him, the Berkefelds, Chamberland F, and other filters; a minute water flagellate was found by Borrel to pass through the coarser filters. Recently, Wolbach and Binger and others have shown that several spirochetes, pathogens as well as saprophytes, pass through Berkefelds V, N, and W, by pressure and by suction. It is seen, therefore, that certain known organisms within the limits of visibility may pass even some of the finer filters. The filtration must be completed within a moderate time, because even bacteria as large as the typhoid bacillus may, in media containing a certain amount of albuminous material, grow, in time, through the filter. The material to be filtered should, furthermore, be greatly diluted and first filtered through filter paper or other coarse filter in order to avoid the clogging action of extraneous material.

If after all the proper precautions have been taken the filtrate is pathogenic, it must be shown that the symptoms are due to a micro-organism and not to a toxin. This may be decided by inoculating a series of animals successively with the filtrate obtained from a previously so inoculated animal.

From our present knowledge, filtrable agents may be divided into two groups: (1) those which have not yet been morphologically demonstrated (ultramicroscopic?); (2) those which are shown to be within the limits of visibility. A third group may be made of the diseases produced by viruses of questionable filtrability.

#### GROUP I.—DISEASES PRODUCED BY FILTRABLE AGENTS OF UNKNOWN MORPHOLOGY.

**Foot-and-mouth Disease.**—This is probably the first disease shown to be produced by a filtrable agent. It is a highly infectious disease of cattle. Other domestic animals may also be attacked. It occurs seldom in man and then among people handling infected cattle or drinking the milk of infected animals. The disease in cattle is characterized by the appearance of vesicles in the mouth and around the coronet of the foot as well as between the toes. Löffler and Frosch, in 1898, discovered that after diluting the contents of an unbroken vesicle with twenty to forty times its volume of water and passing the resulting fluid through a Berkefeld filter, the filtrate contains the virus. This virus remains infectious for some time. One attack of the disease usually produces a certain amount of immunity. Löffler claimed to have produced a serum which has immunizing properties but his work has not been corroborated.

**Mosaic Disease of Tobacco.**—This is the second disease in which a filtrable virus was demonstrated. The disease causes the young tobacco leaves to become devoid of chlorophyll in spots which enlarge, turn brown, and the underlying tissue becomes necrotic. Beijerinck, in 1899, showed that the filtrate from a porcelain filter promptly reproduced the disease on tobacco leaves.

**Cattle Plague (Rinderpest).**—This fatal European and African disease of cattle is characterized by inflammation of the intestinal mucous membrane. The blood is infectious and filtrates of it through Berkefelds and Chamberland F (Nicolle and Adil-Bey, 1902) produce the disease. No organism can be seen. Immunity follows one attack. It can also be produced by a subcutaneous inoculation of bile from an infected animal.

**Yellow Fever.**—The undiluted serum from cases of this disease has been shown by the American commission (1901) and others (see p. 578) to pass the Berkefelds and the Chamberland B filters as clear filtrates, and in this form to be infectious; therefore some forms at least of the specific organism are probably ultramicroscopic.

**Rabies.**—No bacteria have been discovered that are considered as factors. The probability of the Negri bodies being protozoa and the exciting factor is considered in Chapter XLV. The virus of rabies has been shown to be filtrable (Remlinger, 1903). Poor and Steinhardt (1913) showed that gland virus is more filtrable than that from the brain. It may pass through the coarser Chamberland filters.

**Hog Cholera.**—de Schweinitz, Dorset, Bolton, and McBryde demonstrated in 1905 that the blood of hogs suffering from hog cholera con-

tains a filtrable virus which is capable of producing the disease on inoculation into healthy hogs. This virus passes Chamberland B and F filters. It leaves the body in the urine and probably enters another animal through contaminated food. King, Baeslack, and Hoffman found in 1913 a short motile spirochete (*Spirochaeta suis*) in the blood in a series of cases of hog cholera. They think this organism may be a stage of the specific organism which may produce filtrable granules.

This disease is a very fatal and contagious disease of swine characterized by fever and ulcerative enteritis. Immunity follows one attack. An immune serum is produced. Animals are also immunized by a mixture of infected blood and immune serum (sensitized virus). The hog cholera bacillus was earlier supposed to be the cause of this disease.

**South African Horse Sickness.**—This is a warm-weather disease, more common in animals that do not pass the night under cover. The horses are uneasy, have difficulty in breathing, and a reddish froth exudes from their mouths. The temperature rises in the daytime, but has a tendency to drop at night. In severe cases an edematous swelling of the head and neck may appear. MacFadyen succeeded in passing blood serum of a diseased horse (diluted) through the Berkefelds and a Chamberland F, not through a Chamberland B filter.

**Chicken Sarcoma.**—In 1910 Rous discovered a spindle-celled tumor in chickens, which he has reproduced in other chickens by transplantation, by inoculation of dried tumor tissue, and by the inoculation of Berkefeld filtrates from emulsions of tumor cells.

**Novy's Rat Virus.**—Novy has found an extremely virulent filtrable virus from a disease of rats, which passes all filters. He has not yet published full reports.

## GROUP II.—DISEASES PRODUCED BY FILTRABLE AGENTS SHOWN TO BE VISIBLE.

**Contagious Pleuropneumonia of Cattle.**—This malady affects cattle, but not other species. Typically, there is an inflammation of the lungs and the pleura which is invasive and causes necrosis of the diseased parts. Nocard and Roux succeeded in cultivating the organism in collodion sacs placed in the peritoneal cavity of rabbits, using a mixture of serum and bouillon. After two weeks a very faint turbidity appeared in the sacs; coincidentally the fluid became infective. Nocard and Roux described the organisms as minute spheres and spirals just within the limits of visibility. They showed (1899) that the organisms passed the Berkefelds and a Chamberland F, but not a Chamberland B filter. Immunity is produced by a single attack. It has also been produced artificially by the inoculation of cultures or virulent exudates.

**Epidemic Poliomyelitis.**—This is a disease which affects chiefly the central nervous system. It occurs usually in children, and appears sporadically and in epidemics in many countries. In New York, in 1907, there was an epidemic of over 2000 cases and in Texas, in 1912,

there was a large outbreak. During the summer of 1916 one of the largest epidemics known occurred in New York City and the surrounding country. In nature, the virus enters probably through the upper air passages. The chief symptoms of the disease are fever, with or without sore throat, hypersensitiveness followed by paresis and paralysis. The mortality is low. There is usually permanent injury to parts of the motor areas of the nervous system, with resulting deformity. The principal microscopic changes are a marked exudation of polynuclear leukocytes into the lymph spaces and the cerebrospinal fluid. The changes are usually specially marked in the anterior commissure and the cornea of the cervical and lumbar regions of the cord, but the whole nervous system is more or less affected. The bloodvessels may become thrombosed and ruptured. The disease seems to be closely related to, if not identical, with certain diseases manifested by acute encephalitis and bulbar paralysis, *e. g.*, Landry's paralysis. That the disease also bears a marked resemblance to rabies is quite apparent. The finding of the virus in the salivary glands and the demonstration of its filtrability give added evidence of its similarity to rabies.

Until recently nothing was definitely known of the etiology. Now we have a series of studies which seem to have practically cleared up the subject. Landsteiner and Popper (1909) reported the transmission of acute poliomyelitis to apes. They inoculated the spinal cord intraperitoneally and produced typical symptoms and lesions, but did not succeed in transmitting from ape to ape, probably because they used a mild virus. Flexner (1909) and Lewis transmitted the disease from monkey to monkey by means of intracerebral inoculations. Landsteiner and Levaditi (1909) also transmitted it from monkey to monkey. They found that the virus lives some time outside of the body; that the degenerated nerve cells are taken up by phagocytes, and there is an analogy between the lesions of poliomyelitis and those produced by rabies. They demonstrated that the virus is filtrable. Leiner and Wiesner transmitted the disease from monkey to monkey, found young animals more susceptible than older ones, and spinal fluids, blood, and spleen negative. Flexner transmitted the disease by inoculating into the blood or peritoneal cavity, also by the subcutaneous method, and independently found the virus to be filtrable. Landsteiner and Levaditi found the virus in the salivary glands and suggested the saliva, moist or dry, as a source of contagion.

Soon after this (1911) Noguchi and Flexner announced that they had obtained cultures in media similar to that used by Noguchi in cultivating spirochetes (see p. 509). In such media in about five days the pieces of tissue employed become surrounded by an opalescent haze which increases for about five days more, and a sediment gradually forms. Giemsa's stain shows the presence of minute globoid bodies (0.15 to 0.3 diam.) in pairs, short chains, and masses. Cultures were also obtained from the filtered virus. Monkeys inoculated with certain strains of this organism grown for a variable number of culture generations may die with typical lesions of the disease. The authors consider

these bodies the cause of the disease. They further report that the cultures are filtrable. Levaditi states that he cannot obtain the results of Noguchi, but that he obtains evidence of growth by the living tissue method. Rosenow as a result of studies during the recent epidemic states that he has found an aerobic streptococcus which shows in early culture generations specific pathogenicity for the central nervous system, producing paralysis in animals. When grown under anaërobic conditions he says it shows only minute forms. He therefore concludes that it is the cause of the disease and that it is probably the same organism as that described by Flexner and Noguchi.

During this epidemic we also have been studying the etiology of this disease, but our studies are not yet far enough advanced to draw conclusions. We have isolated the Flexner-Noguchi organism in 3 cases out of 50, and definite streptococci in more than half the cases. We can say that the Flexner-Noguchi organism is distinctly different from any aerobic streptococcus studied but we have not yet had enough monkeys to test its ability to produce poliomyelitis in them.

Rabbits and possibly guinea-pigs have also been found to be somewhat susceptible to the virus (Krause and Meinicke, Marks, Rosenau). These authors state that the disease in rabbits does not resemble that in man.

**Immunity.**—One attack seems to give certain immunity. Flexner and Amoss state that, experimentally, the virus inoculated into the blood is capable of being neutralized by intraspinal injections of immune serum.

**Relapsing Fever.**—Todd and Wolbach have found (1914) that *Spirochæta duttoni* may be forced through even the finer grades of Berkefeld filters by a pressure of from fifty to ninety pounds to the square inch. For filtrability of other spirochetes see page 515.

### GROUP III.—DISEASES PRODUCED BY VIRUSES OF QUESTIONABLE FILTRABILITY.

**Smallpox and Related Diseases.**—The details of the disease smallpox are considered in a later chapter. In 1908 Casagrandi reported that the virus was filtrable under pressure through coarser Berkefeld filters. Vaccine virus had already been reported as filtrable by some investigators, and as non-filtrable by others. Our results agree with those of the latter observers. The opinion as to the filtrability of the other "pox" diseases is not unanimous.

**Dengue.**—Ashburn and Craig claim to have reproduced dengue in susceptible individuals by a procedure similar to that employed in yellow fever. The virus passes a Berkefeld filter. The intermediary host in natural infection is claimed by them to be *Culex fatigans*.

**Measles.**—This very definite infectious exanthematous disease still remains among those of unknown etiology. Both cell inclusions (Field, Ewing, and others), bacilli and cocci have been reported as having an etiological relationship. The reports have not been corroborated. In 1905 Hektoen produced measles in two human cases by the inoculation

of blood drawn from an infected case at an early stage of the disease. Anderson and Goldberger, in 1911, inoculated monkeys with measles blood and demonstrated the virus in the blood of the inoculated animals and in the secretion of the upper air passages. They also demonstrated the period of infectivity of the blood. They reported positive results with Berkefeld filtrates. Tunncliffe has just reported finding a small anaërobic coccus in the blood of 42 out of 50 cases of measles.

**Scarlet Fever.**—Scarlet fever is an acute febrile, highly infectious disease, characterized by a diffuse, punctate, erythematous skin eruption, accompanied by catarrhal, croupous, or gangrenous inflammation of the upper respiratory tract and by manifestations of systemic infection. The disease was probably known long before the Christian era, but the present name does not appear until the time of Sydenham (1685), who differentiated the disease from measles. It is very generally disseminated, but is much more common in temperate climates than in the tropics. The specific exciting factor is thought by some to be a streptococcus, of the *Streptococcus pyogenes* type, but the evidence in favor of this view is very slight (see *Streptococcus pyogenes*).

Mallory (1914) found certain bodies occurring in a series of forms in and between the epithelial cells of the epidermis and free in the superficial lymph vessels and spaces of the corium. He gave the name *Cyclasterion scarlatinale* to these bodies in consequence of the frequent wheel and star shapes of the rosette. Field and others think that these bodies are not specific germs. Recently Mallory reports the finding of a small pleomorphic bacillus which he thinks may be the specific cause. The reports that a filtrable virus is found lack corroboration.

**Trachoma.**—This condition has already been considered in a previous chapter. Bertarelli reported that he was able to produce a specific filtrate. Nicolle also reported positive results with filtrates. The descriptions of the disease, however, are too vague to allow an opinion as to the truth of these reports.

**Other Diseases said to be due to Filtrable Viruses.**—Several other diseases of less importance have been listed as belonging to those produced by a filtrable agent. When a well-known disease such as epidemic cerebrospinal meningitis is reported as being due to a filtrable virus, much corroboration is needed before we can accept the statement as a fact.

Recently Kruse corroborated by Foster reported that a filtrable virus is obtained from common colds which would reproduce the disease.

#### OTHER DISEASES OF UNDETERMINED ETIOLOGY.

**Rocky Mountain Spotted Fever.**—This is an acute infectious disease characterized by fever and a more or less hemorrhagic eruption. Some years ago Wilson and Chowning thought they found a protozoan in the blood similar to babesia in Texas fever. Their findings have not been corroborated. Their investigations proved, however, that rabbits are susceptible to the disease and that a tick of the genus *Dermacentor* probably carries the infection. Then Ricketts and Gomez made some very interesting studies on the disease. They found that guinea-pigs

and monkeys are susceptible as well as rabbits, and they further found that in guinea-pigs and monkeys an attack of spotted fever produces a strong active inherited immunity characterized by a serum with high protective but low curative power, and that the production of the serum in the horse with the use of serovaccination in man may give practical results. They found a moderate number of diplococcoid bodies in the blood of infected guinea-pigs and monkeys, and fewer in man. They found that the virus is transmitted by the infected female tick to her young through the eggs. If the larvæ from these eggs are allowed to feed upon normal guinea-pigs, these animals come down with the disease. Immense numbers of these apparent organisms are found in affected eggs and none were found at first in normal eggs. Afterward Ricketts found a few, but he thought these might be an avirulent species of the same organism. The salivary glands, alimentary sac and ovaries of infected female ticks are swarming with these bodies, while normal ticks seem to have none. Lastly, Ricketts found that these bodies agglutinate with specific serum, 1 to 300 dilution. Recently Wolbach has corroborated some of these findings and demonstrated a similar organism in characteristic lesions of experimental animals bitten by infected ticks. Frick recently reported the finding of small bodies in centrifuged blood cells, which he did not classify.

**Typhus Fever.**—The occurrence of this infectious disease in epidemic form had disappeared from civilized lands until the recent war broke out. The fact that the body louse may transmit the disease helps explain why it has always been classed as a filth disease. It still occurs endemically in parts of Europe and North and South America. In Mexico it occurs in epidemics where it is known as *Tabardillo*. In New York it occurs occasionally in mild form under the name of Brill's disease. Brill thought it was a new disease, but Anderson and Goldberger have shown that typhus fever and Brill's disease are the same. Nicoll and Krumwiede observed four cases which clinically verified these findings. The disease is characterized by high temperature and a petechial rash.

Nicolle (1909) showed that the old world typhus can be transmitted to the chimpanzee and from this to the macacus with typical eruption in each case. He also showed that the disease is transmitted by the ordinary body louse (*Pediculus vestimenti*). Anderson and Goldberger (1909) were the first to transmit typhus fever of Mexico (tabardillo) to monkeys. They were able to transmit directly from human beings to the macacus and capuchin and from monkey to monkey. Ricketts and Walker (1910) also found that the macacus was directly susceptible to the disease. They based their diagnosis chiefly upon a rather indefinite fever and in most cases somewhat distinct symptoms of illness. They also found that the monkey may pass through an attack of typhus so mild that it cannot be recognized clinically, but it results in immunity. The immunity test is a reliable proof of the previous occurrence or non-occurrence of typhus at least within a period of one month. They found that typhus was transmitted to the monkey by the bite of the louse. They further state that in stained preparations

of blood of patients taken from the seventh to the twelfth days of the disease they invariably found a few short bacilli similar to those which belonged to the hemorrhagic septicemia group. In moist preparations they saw similar forms in all cases. No motility was observed. No cultures could be obtained. They examined the dejecta of many lice and found similar bodies in large numbers in infected lice and occasionally in non-infected lice. *Pediculus capitis* may also transmit the disease.

Recently, Plotz reported the isolation in pure cultures of a Gram-positive pleomorphic anaërobic bacillus from cases of typhus and of Brill's disease. His report is given on page 451.

Attempts at filtration by Ricketts and Wilder, Anderson and Goldberger and Nicolle, Connor and Wilder showed that filtered blood inoculated into monkeys is probable non-filtrable. Olitzky has just gone over this work and added experiments of his own which seem to prove that the virus is non-filtrable.

**Chicken-pox.**—Kling claims to have been able to vaccinate against this contagious disease with the clear contents of a fresh vesicle in as early a stage as possible. He inserted the point of a sterile lancet into such a vesicle and then into the patient's skin, repeating six times. This work was corroborated by Rabinoff.

**Mumps.**—Comparatively few studies have been made of this infectious disease, probably because of its low mortality. By puncturing the parotid gland, Laveran and Catrin obtained a diplococcus which stains easily, is Gram-negative, and grows on ordinary culture media. No satisfactory specific studies have been made of this organism.

Hess, in 1915, used serum from recovered cases of mumps in a children's home as a protective measure, with the result that none of the 17 so inoculated came down with the disease on exposure.

**Pellagra.**—This disease has been much studied recently. The theory that it is due to the ingestion of damaged corn received a check from the work of Siler, Garrison and MacNeal (1913) who stated that they were unable to get evidence to support this theory. They consider the disease a specific infection caused by unknown means. Since then the work of Goldberger and others added much to the evidence that the disease is due to a deficiency of certain substances in the diet which may be corrected by including in the diet suitable proportions of fresh animal and leguminous protein food.

**Verruga peruviana**, a South American disease has been shown by Strong and his co-workers to be a disease distinct from oroya fever (see p. 552) and to be produced by an unknown virus transmitted, according to Townsend, by the bite of a gnat (*Phlebotomus verrucanamun*).

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## CHAPTER XXXVII.

### FLAGELLATA.<sup>1</sup>

THE flagellates that are pathogenic to man belong chiefly to the genera *Trypanosoma* and *Leishmania*, but certain other genera are found occasionally in man which may be mentioned because of the possibility of their becoming pathogenic. According to the classification of Calkins (1908) the flagellates parasitic in man are from three orders, the *Monadida*, the *Heteromastigida*, and the *Polymastigida*; according to that of Doflein and Koehler (1913), they are from two, *Protomonadina* (one to three flagella), and *Polymastigina* (four to eight flagella). Six families, according to the morphology of the flagella, are distinguished, three of which contain forms parasitic for man, namely, *Cercomonadidae* (one flagellum and no blepharoplast), *Trypanosomidae* (one flagellum, a blepharoplast, and an undulating membrane), and *Bodonidae* (two flagella).

Under the *Trypanosomidae*, which include most of the pathogenic forms, are placed the genera *Leptomonas*, *Herpetomonas*, *Leishmania*, *Trypanosoma*, and *Schizotrypanum* and *Endotrypanum*. Under the *Bodonidae* are placed the genera *Bodo* and *Prowazekia*, and in the order *Polymastigina* the genera *Trichomonas* and *Lambliia* have been found in human beings.

Hartmann puts the *Trypanosomata*, with other blood parasites, in an order, the *Binucleata*, and makes the *Spirocheta* an appendix of this order. According to this arrangement the *Hemosporidia* are taken from the *Sporozoa* and placed in the *Binucleata* with the *Trypanosomata*. The malarial organisms supposedly lose through their parasitism many of the characteristics ascribed to this order.

**Material and Methods for Study.**—A number of flagellates (*Bodo*, for instance, see p. 518) are found in the large intestine of the lower animals. The toad, the grass lizard, and the guinea-pig may contain some interesting forms. As these forms are easily obtained and remain alive a long time outside of the body, they are well fitted for class study.

The feces are obtained by pressing lightly over the anus of the animal, or if the whole intestinal tract is to be examined, by sacrificing the animal and dissecting out the parts wanted. The material is placed in a clean watch-glass and thinned if necessary with physiological salt solution. Hanging drops may be made in physiological salt solution or in such a solution made a little thick by the addition of gelatin in order to retard the motion of the flagellates somewhat so they may be better studied.

Permanent preparations may be made according to directions given in Part I. As most of the pathogenic members of this group may be difficult to obtain in the living condition at any stated time, they must be studied by students principally in stained smears and sections.

If one can obtain rats infected with *Tr. lewisi*, others with one or more pathogenic forms still others with *Spirocheta obermeieri*, the infecting organisms

<sup>1</sup> See Part I for general description of Protozoa.

can be kept alive by frequent reinoculation of the heart's blood, subcutaneously or intraperitoneally into the fresh animal, or cultures may be carried on (see below). But this is an expensive and tiresome work in those laboratories where such work is not being carried on, and generally one must rely on the permanent preparation. In the development in the second host one must also study the stained specimens in the great majority of instances.

The fresh specimens of blood are obtained from the tail tip of the rat, or the ear of the dog; they may be examined, after dilution with physiological salt solution, in the hanging drop, or in a drop spread under a cover-glass and ringed with vaselin. For permanent preparations films of the blood are spread, fixed, and stained in the usual way; Giemsa's method of staining (p. 82) is very satisfactory.

For section work of the various organs the fixatives and methods given on page 84 may be used. Special methods are given under each organism.

**Artificial Cultures of Blood Flagellates** (see p. 499).—These, according to Novy and MacNeal, may be made on a culture medium consisting of a mixture of ordinary nutrient agar with variable amounts of fresh defibrinated rabbit or rat blood. The best all-round results are obtained with equal parts of blood and agar. The agar is melted and cooled to 50° C., then the blood is added and thoroughly mixed. The tubes are inclined until the medium stiffens, when they should be inoculated at once with blood or other infected material containing living trypanosomes. The surface of the medium should be very moist, so water of condensation may form. Generally evidence of growth may be observed in three or four days.

### CERCOMONAS.

The members of this genus are round or oval flagellates with a long anterior flagellum and a more or less pointed posterior extremity which is sometimes ameboid. The vesicular nucleus is situated anteriorly, and passing through the organisms from flagellum to posterior extremity is an axial elastic fibril (Fig. 169, a). Division into two daughter forms has been observed.

A number of cercomonada, none of them well studied, have been observed in different animals as well as in man. They are very numerous in stagnant water.

*Cercomonas hominis* (Davaine, 1854) was observed in the dejections of a cholera patient by Davaine. The body is 10 $\mu$  to 12 $\mu$  long and pear-shaped, pointed posteriorly. The flagellum is twice as long as the body. Davaine also reported a smaller form in the stools of a typhoid patient. Other observers have noticed similar forms in human stools, some associated with "*Amoeba coli*." Similar forms have been seen also in an echinococcus cyst of the liver, in the sputum from a case of lung gangrene, in the exudate of a hydropneumothorax, and a few times in the urine. They are all probably harmless invaders.

### LEPTOMONAS, HERPETOMONAS, AND CRITHIDIA.

Certain flagellates found in the digestive tract of mosquitoes, flies, and other insects are very similar to trypanosomes. Among them several species have been recognized, but they need to be more fully studied in order to determine their definite relationship to each other and to the genus trypanosoma. *Leptomonas* is described as having a single flagellum directed forward and arising near a blepharoplast situated in the anterior part of the cell. *Herpetomonas* is distinguished from *leptomonas* by a flagellum containing two filaments and by a delicate filament extending from the blepharoplast toward the posterior end. *Crithidia* has a rudimentary undulating membrane. The distinctions

between these three genera and the genus *Trypanosoma* which have been recognized are: (1) the former contain no undulating membrane or only a rudimentary one, and (2) their centrosome or blepharoplast usually lies at the side of, or anterior to, the nucleus instead of posterior to it, as in *Trypanosoma* (Fig. 168).

These distinctions, Novy claims, may disappear in the cultural forms of the three genera, when trypanosomes may show a rudimentary undulating membrane and an anterior blepharoplast. His caution in regard to confusing these insect flagellates with developmental stages of vertebrate blood parasites should be remembered.

The Leishman-Donovan bodies found in kala-azar are closely related to the genus *Leptomonas*. They are considered a separate genus, *Leishmania*.

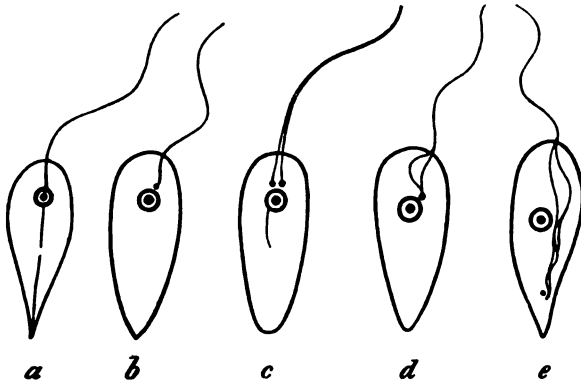


FIG. 168.—Schematic drawings of flagellates belonging to the trypanosomedaë, showing differential points: a, *Cercomonas*; b, *Leptomonas* and *Leishmania*; c, *Herpetomonas*; d, *Crithidia*; e, *Trypanosoma*.

### LEISHMANIA (LEISHMAN-DONOVAN BODIES AND ALLIES).

Certain fevers of severe malarial-like types known in different sections of the tropics by different names (dum-dum fever, cachexial malaria, kala-azar) have been shown to have a casual relationship by the finding of similar protozoön-like bodies in the lesions. These bodies were first minutely described by Leishman, in 1903, as being present in certain cells in the spleen of cases occurring in India, called by him dum-dum fever. He considered them as possibly trypanosomes, but did not name them. Later in the same year Donovan described similar bodies in cases of what he called malarial cachexia. The bodies were first called the Leishman-Donovan bodies; then Laveran and Mesnil who examined Donovan's preparations and considered the organisms similar to those causing Texas fever in cattle, called them *Piroplasma donovani*. Ross, however, thought they constituted a distinct genus which he called *Leishmania*. This genus is now accepted, hence they are known as *Leishmania donovani*. Rogers and Patton place them with the genus *Herpetomonas*, but until we know more of the limits of variation of all these forms it seems best to make them a separate genus. They have since been found in different parts of India, in China, Tunis, Algiers, Arabia, Egypt, South Africa, Italy, Greece,

and Portugal. Wright, in this country, has reported in a case of tropical ulcer, or Delhi boil, from an Armenian immigrant, bodies which, according to his excellent photographs (Fig. 169) and description, must be identical with, or very closely related to, Leishman's bodies. On account of the different pathological conditions in which they are found, however, they are classed as a different species, *Leishmania tropica* Wright. The form found in infantile splenomegaly is considered another species, with the name *Leishmania infantum* Nicolle. Darling described an organism resembling that of kala-azar found in a fatal disease of tropical America. Though the organism, he says, resembles *Donovani*, he thinks it has enough points of difference to be placed in a different

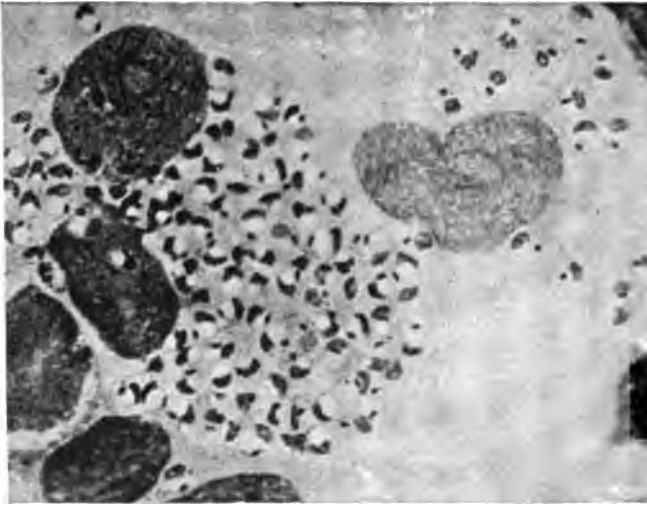


FIG. 169.—*Leishmania tropica* in a case of tropical ulcer. Smear preparation from the lesion stained with Wright's Romanowsky blood-staining fluid. The ring-like bodies with white central portions and containing a larger and a smaller dark mass are the microorganisms. The dark masses in the bodies are stained a magenta, while the peripheral portions of the bodies in typical instances are stained a pale robin's egg blue. The very large dark masses are nuclei of cells of the lesion.  $\times 1500$  approximately. (After Wright.)

genus; therefore he gives it the name *Histoplasma capsulatum*, and calls the disease histoplasmosis. He says it differs from *Donovani* in the form and arrangement of its chromatin nucleus and in not possessing a chromatin rod. It has a refractile achromatic capsule.

**Morphology.**—The bodies as seen in the cells of the host are circular to elliptical in shape, from  $2\mu$  to  $4\mu$  in diameter, and contain two nuclei, a large oval one at one part of the periphery and a small circular or rod-shaped one (blepharoplast) near or at the opposite part of the periphery. This smaller body stains more intensely than the larger one, while the cytoplasm of the parasite stains very dimly, sometimes showing only a faint peripheral rim. Near the rod-shaped nucleus may sometimes be seen a minute granule or rod which is the rudiment of

the flagellum. Any nuclear and cytoplasmic staining methods will bring out these points in Zenker-fixed material. Smears stain well by Wright or the Nocht-Romanowsky methods. It was not until these organisms were cultivated outside of the body that their relationship to the flagellates was established (Fig. 170 and Plate IV, 1, Fig. 1, B).

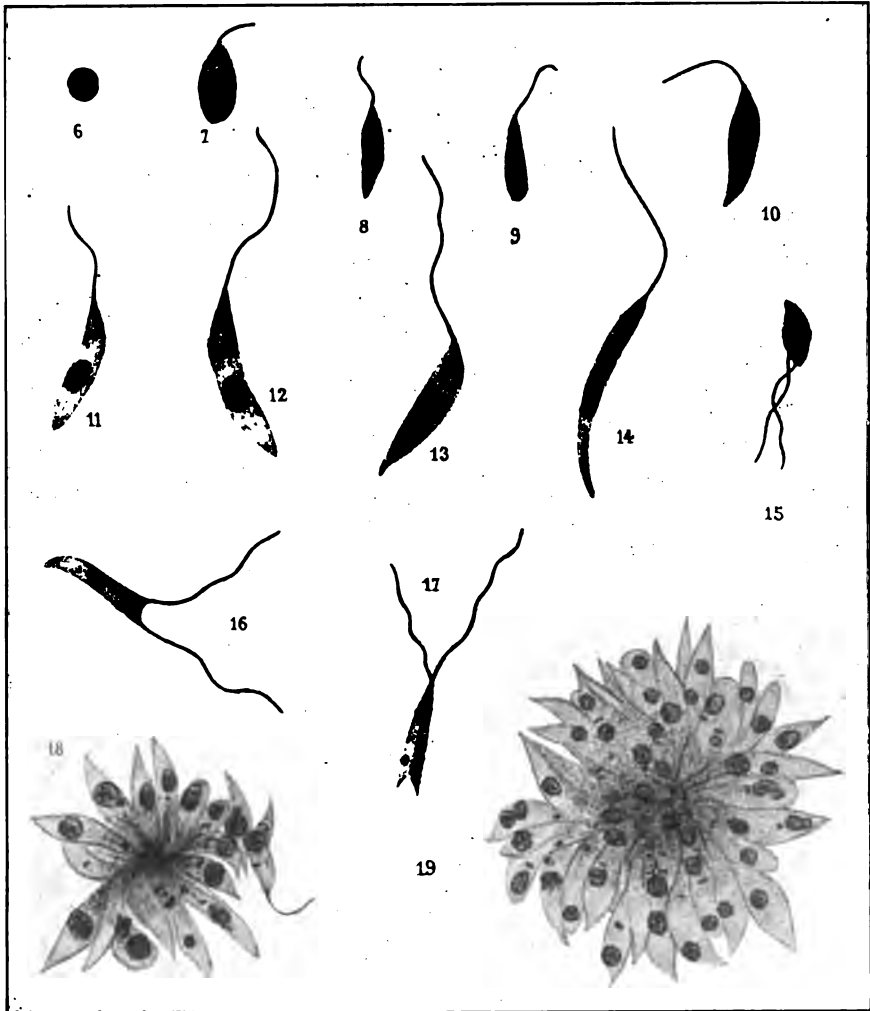


FIG. 170.—Cultural forms of *L. infantum*, showing the flagellate type. (After Nicolle.)

**Site in Body.**—The bodies have been found in large endothelioid cells in the spleen, liver, bone marrow, lymph nodes, kidney, lungs, testes, skin, muscles, intestinal ulcers and in the leukocytes in the peripheral blood. In this last situation they are only found in appreciable numbers in advanced cases.

The large cells containing the parasites are supposed by Christophers to be the endothelial cells from the finest capillaries. Donovan states that he found small forms in the polynuclear leukocytes of the peripheral circulation when the temperature was above 103°. Marshall found them chiefly in the mononuclears. Rogers was able to demonstrate them more easily in the peripheral circulation by centrifuging the blood and examining the leukocytic layer. Nicolle and Conte and others obtained them in the mononuclears found in the fluid of blisters produced experimentally on the skin.

**Cultivation.**—Rogers has grown abundant pure cultures of the bodies in a slightly acid citrated blood medium at 20° to 22° C. Nicolle and later Novy have shown that *L. infantum* is pathogenic for dogs and that cultures may be obtained with comparative ease from the infected animals (Fig. 170). Nicolle has also cultivated *L. tropicum*.

In these cultures the rounded organism elongates, the flagellum develops, and the Leishman-Donovan body becomes a flagellate, like the *Leptomonas* (Fig. 168).

**Insect Carriers.**—Rogers and Patton have shown that the bed-bug may transmit the disease, and Patton has demonstrated the development of the organism up to the fully flagellated stage in the gut of this insect. The Sargents have shown that *L. infantum* may be transmitted by the dog flea.

**Effect on Human Host.**—The pathological changes are those following the degenerations, subsequent to the growth of the organisms in the large mononuclear cells. The *symptoms* in the cases of general infection are: (1) Very much enlarged spleen and less enlarged liver; (2) progressive anemia with peculiar dark, earthy pallor of skin (kala-azar), progressive emaciation, and muscular atrophy; (3) long-continued, irregularly remittent, and intermittent fever (97° to 104°); (4) hemorrhages, such as epistaxis, bleeding from gums into subcutaneous tissue, producing purpuric eruption; (5) transitory edemas of various regions. There are often complications, such as congestion of lungs, dysentery, and cancrum oris. The blood shows practically no loss of red blood cells, but a diminution of hemoglobin; there is a decrease in the leukocytes, principally polynuclears, giving a relative increase of mononuclears.

Negative points which help in the diagnosis are: absence of malaria, no typhoid or Malta fever reaction, resistance to medication, quinine, as a rule, having no effect though in early cases and with large continued doses a few good results have been reported. Puncture of spleen or liver with the finding of Leishman-Donovan bodies makes the diagnosis certain. Sometimes the bodies may be found in the peripheral blood.

The duration of the disease is from a few months to several years. The percentage of deaths in systemic infections is great; in some forms of the disease at the height of an epidemic it may reach 98 per cent. Strangers from non-tropical countries are specially susceptible. The infection in children known as splenomegaly is similar to that in the adult.

The local disease known as Delhi or Aleppo boil or tropical ulcer is a comparatively non-dangerous circumscribed chronic ulcer in which the endothelial cells contain the organisms in large numbers (Fig. 169). Recovery is followed by marked immunity.

**Complement-fixation.**—Makkas and Pappasoterion state that a specific antigen gives positive results with both kala-azar and syphilis, but a syphilitic or a non-specific antigen gives positive results only with syphilis, therefore both should be used.

**Prophylaxis.**—Segregation and perfect cleanliness, especially in regard to bed-bugs and fleas, are recommended as the best means of eradicating the disease.

**Uta.**—A South American disease has been shown by Strong and his co-workers (1913) to be due to a species of *Leishmania*. The flagellate stage of the organism was obtained and animals were successfully inoculated from a human case.

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## CHAPTER XXXVIII.

### TRYPANOSOMA.

**Pathogenic Forms.**—Very many species of trypanosoma have been described, and the number reported as distinctly pathogenic is increasing. Two of the latter are known to be pathogenic for man; a closely related form that was described by Chagas in 1909 is made a new genus. The table on page 495 gives a list of the better-known forms pathogenic for mammals with their chief differential characteristics. They are divided by Laveran into three groups according to the different characters of the flagellum. Their general characteristics and broader classifications are given in Part I. (See also Plate IV, 1, Fig. 2.)

**Historical Note.**—The first species of trypanosome studied with any degree of fulness is the comparatively non-virulent *T. lewisi*. It was probably first seen in the blood of the rat in 1845, but was not well described until 1879, when Lewis studied it more fully. Since then it has been studied by many observers. It is found in the blood of from 2 to 3 per cent. of wild rats throughout the world.

The first of the more pathogenic trypanosomes was discovered by Evans in the blood of East Indian horses suffering from surra, but it was not well studied until 1893, when Lingard's important work on surra led, in a way, to all the subsequent work on diseases caused by trypanosomes. The next year a trypanosome was discovered by Bruce in the blood of horses and cattle suffering from *nagana* in Zululand and other parts of Africa. Bruce further demonstrated the important fact that the disease was transmitted by the bites of flies, the tsetse flies (*Glossina*). Announcements of other pathogenic trypanosomes in different parts of the tropics quickly followed. In 1896 Rouget found that *dourine*, a disease of equines in Algiers and South Africa, was caused by a trypanosome (*T. equiperdum*). Then the South African disease of horses, called *mal de Caderas*, was shown by Voges to be due to a similar flagellate, while in 1902, Theiler found a variety of trypanosome in the blood of cattle in the Transvaal suffering from the disease called *galziekte*, or gall sickness. The number of trypanosomes found in the tropics is constantly increasing—both pathogenic and non-pathogenic forms.

Man was thought to be comparatively immune to trypanosomes until the important discovery was made that trypanosomes are the specific cause of a definite disease known as *sleeping sickness*, which occurs chiefly in the African negro. In 1898 Nepveu reported having found trypanosomes in the blood of 6 out of more than 200 cases of human beings examined for malarial organisms and in a seventh case which was apparently in good health. The eighth case is reported by Dutton in 1901. The tenth and eleventh cases were published by Manson in 1902. Bröden published 2 more cases, and Baker 3. In 1904 Castellani stated that the cause of sleeping sickness of the negro is a trypanosome. He found trypanosomes in the centrifugalized cerebrospinal fluid of 20 out of 34 cases of this disease. His work has been fully corroborated. The trypanosomes found in these cases resemble each other; they are therefore included under the same name, *Trypanosoma gambiense* Dutton. A similar form found by Stephens and Fantham in 1911 in cases of sleeping sickness in

DIFFERENTIAL CHARACTERISTICS OF MORE IMPORTANT TRYPANOSOMES PATHOGENIC FOR MAMMALS (MODIFIED FROM YORK AND BLACKLOCK).

Groups	Sub-groups.	Species.	Date discovered	Where first found.	Size in microns.				Other characteristics.	Pathogenesis.			Invertebrate host.	
					Length.	Breadth.		Vet-erbrate host.		Name of disease.	Laboratory animals.	Name.	Place of development.	
						Max.	Min.							Max.
I. Always free flagel-lum.	1	T. lewisi Kent	1881	Throughout world	38	10	3.0	1.5	Very motile, nucleus at anterior and middle third, blepharoplast rod-shape-.....	Rats	Trypano-somiasis	Rat slight	Rat fleas (Cten-ophthalmus) and lice (Hem-matopinus)	Intestines.
		T. evansi Steele	1885	India	34	18	2.0	1.5	.....	Equidae, cat-tle, camels	Surra	Acute	Stomoxys (fly)	Unknown.
	2	T. brucei Plimmer and Bradford T. hippicum Dar-ling	1899 1910	Zululand Central America	34 28	18 18	2.5 3.0	1.5 1.5	Post. end usually bluntly rounded.....	Equidae, cattle Equidae	Nagana Trypano-somiasis	Acute Acute	Glossina .....	Gut and sali-vary glands. Unknown.
	3	T. equiperdum Doflein	1901	North Africa	35	16	3.0	1.5	.....	Equidae	Dourine	Acute	Transmitted by coitus.	Proboscis.
	4	T. vivax Ziemann	1905	Cameroon	31	16	3.0	2.0	Very motile, club shaped	Equidae, cattle	Trypano-somiasis	Sleeping sickness	Glossina	Proboscis.
II. Never free.	5	T. theileri Laveran	1902	German East Africa	70	25	5.0	2.0	Very large	Equidae, cattle	Sleeping sickness	Slight	Conorhinus	Gut and sali-vary glands.
	6	T. (Schisotrypan-um) cruzi Chagas	1909	Brazil	Average	20.0			Dividing forms not seen in blood, blepharoplast large	Man, monkey	Trypano-somiasis	Acute	Glossina	Proboscis.
	7	T. congolense Bor-den	1904	Congo	19	8	Aver-age	1.5	.....	Equidae, ruminants	Trypano-somiasis	Acute for monkeys and pigs	Glossina	Proboscis.
III. Free or not.	8	T. simiae Bruce	1912	Nyasaland	23	12	..	..	.....	Monkeys	Trypano-somiasis	Acute	Glossina	Gut and sali-vary glands.
	9	T. gambiense Dut-ton	1902	Gambia	39	13	..	..	.....	Man	Sleeping sickness	Acute	Glossina	Gut and sali-vary glands.
	10	T. equi Blacklock and Yorke	1913	North Africa	36	14	..	..	Posterior nuclear forms	Equidae	Sleeping sickness	Acute	Transmitted by coitus.	Gut and sali-vary glands.

Rhodesia has been given another specific name, *T. rhodesiense*, chiefly because of the characteristic action in experimental animals.

Chagas, in 1909, states that a trypanosome which he had discovered in a small monkey (*Callethrix hapalepeneceolata*) is the cause of human infection in Rio de Janeiro. Because of its ability to grow in the tissues of infected animals it is classified by some as a new genus—schizotrypanum. It is carried by an hemiptera, genus *Conorhinus*. The flagellate is small with a large blepharoplast (kinetodonucleus). It grows on blood agar readily and infects laboratory animals easily. Chagas reports developmental forms in the monkey's lung and in the gut of the fly.

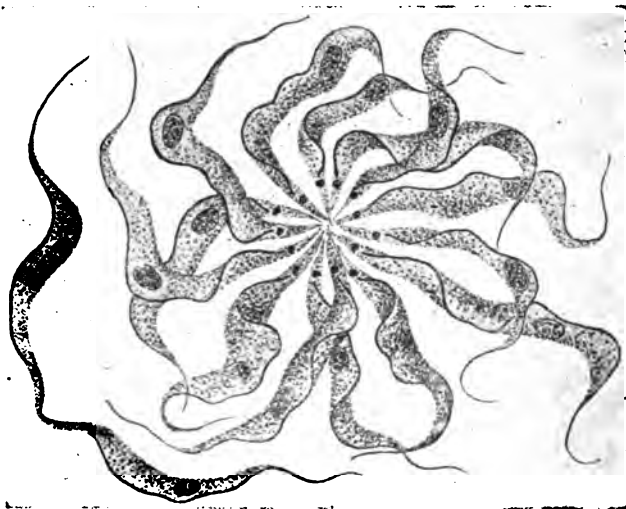


FIG. 171.—Agglutination of *Trypanosoma lewisi*. (Laveran and Mesnil.)

**Comparative Characteristics of the Different Species.**—The form changes of the same species in the same host are so varied that few have been found absolutely characteristic of a single species, and, as physiological properties alone are not considered final in species classification, we cannot be sure that all of the organisms in this group described as separate species are so until more of the complete life histories are known. Until then each new form found with distinct physiological properties, though apparently morphologically similar to others, may expediently be considered a new species.

As an example of the difficulty of deciding whether or not a trypanosome found is a new species, the history of *T. rhodesiense* may be taken. All of the points brought forward by Stephens and Fantham, the discoverers, as evidence of a new species have been combated by others; and its relation to *T. gambiense* on one side and *T. brucei* on the other is an open question. At present the points are in favor of its being the same as *T. brucei*.

**Morphology.**—**Size.**—The variations recorded in the *dimensions* of the species we are considering may be seen by glancing at the above table. The trypanosomes pathogenic for man (*T. gambiense* and *T. rhodesiense*) have the smallest average size of the group. With the exception of

*T. theileri* and *T. transvaaliense* (not given in table), which are much larger than any other of these forms, the variations in size of the different species are not so marked as they are in the same species under different conditions.

**Shape.**—In shape, though all follow the type, each species varies greatly according to conditions of growth and multiplication. At times they may be slender and worm-like, at others they may be so short and thick as to be almost round. *T. lewisi* has the posterior (aflagellar) end often thinner and more pointed than the other species. *T. evansi* is generally a little longer and thinner than *T. lewisi*, while *T. brucei* has a more rounded aflagellar end than either, and is generally broader, more club-shaped (Plate IV, Figs. 1, 2).

**The Cytoplasm.**—The cytoplasm differs slightly in the different forms. *T. lewisi* is relatively free from chromatoid granules, while *T. brucei* has usually many. Myoneme fibrils have been demonstrated in some species and probably all contain them. An oval vacuole has been seen in some species.

**The Nuclear Apparatus.**—The nuclear apparatus is essentially similar in all forms. The two nuclei (tropho- and kinetonucleus) vary somewhat in position and size in the different species and at different stages in the same species. In *T. theileri* and in young forms of *T. lewisi*, both nuclei lie close together near the centre of the organism. In *T. lewisi* the trophonucleus is situated more anteriorly than in the other species. In *T. rhodesiense* the nucleus is often posterior to the centre, especially in experimental animals.

Many variations from the type forms are seen. Some are no doubt degeneration and involution forms. Three forms, however, which are more or less constantly seen in all the species have been interpreted as definite phases in the life cycle. These forms were first described by Schaudinn in *T. noctuae*, and were interpreted by him and since then by others as male, female, and indifferent form. The male cells are smaller, more hyaline, and more free from granules than the female. The nucleus of each sexual cell rides itself of male and female chromatin, respectively. The indifferent cell, on the contrary, has a complete nucleus. Opinions differ as to Schaudinn's interpretation being the correct one. More research is needed before we can arrive at a definite conclusion.

**Motility.**—The first thing noticed on examining a fresh hanging drop of blood at a magnification of 100 to 300 diameters is active movements of the red blood corpuscles in certain areas, and, on carefully focussing over one of these areas, the rapidly wriggling worm-like organism may be seen. As the movements become slower, the flagellum may be seen swaying from side to side and the wave-like movements of the undulating membrane are quite discernible. Movement is twofold: (1) progression with an auger-like motion effected by the undulating membrane assisted by the flagellum; (2) contractions of the body assisted no doubt by myoneme-like structures. Relatively, *T. lewisi* is most active and *T. brucei* least. Motility soon ceases outside of the body, continuing longer if the organism has been kept in the ice-box than at higher temperatures. Aflagellar forms, sometimes ameboid, have been frequently described in the blood of mammals.

**Reproduction.**—The usual method of multiplication is binary longitudinal fission. In several species a rosette-like segmentation has also been observed. Longitudinal fission begins usually with division of the kinetonucleus, then of the trophonucleus and cytoplasm; but this order of division seems to be quite variable. The flagellum often appears to be dividing first, and probably division always starts with the centrosome-like basal granule of the flagellum. In many cases a new flagellum seems to be formed instead of division of the old one. The details of division have not been frequently studied, but it is probable that both nuclei divide by a primitive mitosis. During division the kinetonucleus generally moves near the trophonucleus. Generally the fission is equal, but occasionally the daughter trypanosomes may be quite unequal in size. This is notably the case in division of *T. lewisi* where the cytoplasm may divide so unequally that the process may be compared to budding. The resulting small parasites have at first no undulating membrane, hence they resemble somewhat *Leptomonas*. These young forms may divide several times in succession, producing smaller and smaller fusiform parasites. As a result some forms are so small that they can only be seen when agglomerated or in motion (Schaudinn). The question as to whether trypanosomes undergo phases of development in their invertebrate hosts has been widely studied. Prowazek has described sexual forms of *T. lewisi* in the body of the louse. The subject, however, requires further research for convincing evidence.

**Insect Carriers.**—The trypanosomiasis of vertebrates is transmitted by blood-sucking insects (dourine is possibly an exception). Bruce (1894) first showed that *T. brucei* was conveyed by the fly *Glossina morsitans*. Since then other varieties of flies also have been shown to spread the disease (table, p. 495). Among them *Glossina palpalis* (Fig. 172) is supposed to be the chief agent in transmitting human trypanosomiasis. These flies bite by day and in full moonlight. The infective period of the insects after they have bitten a sick animal is variable. Bruce found living trypanosomes in the proboscides of the flies up to forty-eight hours. Up to one hundred and eighteen hours he found them in the flies' stomachs, after one hundred and forty hours he found the stomachs empty, and what appeared to be dead parasites in the excreta. Klein and others have since found that after a certain number of days (usually from twelve to twenty-five) a small percentage of the biting flies which were fed sufficiently on an infected animal become infective again. This is due to the fact that the trypanosomes have developed in the gut and have passed to the salivary glands of the fly. They remain in the glands probably during the life of the fly, and continue to be infective (Bruce, Hammerton, Bateman, Mackie, and others).

Dourine in horses is usually communicated during the act of coitus. Surra may be transmitted to dogs by eating infected animals. The rat trypanosome is conveyed by contaminative infection through the dejecta of the fleas or lice when biting a new host.

**Cultivation.**—Novy and MacNeal were the first (1903) to cultivate trypanosomes in the test-tube. They have grown *T. lewisi* through many culture generations extending over several years. At the last tests made the parasites were as virulent as at the beginning. The culture medium used in their work was the condensation fluid from slant tubes of ordinary nutrient agar containing variable amounts of fresh defibrinated or laked rabbit or rat blood. The best results were obtained with a mixture of equal parts of blood and agar. At room temperature the growth is slower but surer than in the thermostat. A culture at room temperature retains its vitality for months; thus in one case the trypanosomes were alive after three hundred and six days. Novy and MacNeal also cultivated *in vitro* *T. brucei*, *T. evansi*,



FIG. 172.—*Glossina palpalis*, carrier of the human trypanosomiasis caused by *T. gambiense*.  
× 4. (Kolle and Wassermann.)

and various bird trypanosomes. The latter they found especially easy to cultivate, while the former are much more exacting in their requirements than is the *T. lewisi*. They require two parts of blood to one of agar, and growth is best at 28° C. The primary cultures are not transferred until the end of three weeks. These primary cultures are not always virulent for animals, but subcultures regain their virulence. Mice and rats die in six to fourteen days after inoculation. Guinea-pigs have a relapse in two to ten weeks.

The great majority of trypanosomes experimented with since the initial investigations, have been found by various workers to be cultivatable, with more or less ease. *Trypanosoma gambiense*, however, seems to be more difficult to cultivate (Thompson and Sinton).

**Effect on Vertebrate Host (Pathogenesis).—Lower Animals.**—Many of the lower vertebrates have become, through mutual toleration, natural hosts of the trypanosome. It is probable that each pathogenic trypanosome has an indigenous wild animal as natural host and that in this way the supply to strange mammals coming into the vicinity is kept up. These strange animals, being unaccustomed to the native trypanosomes, often succumb to the infection.

**Symptoms.**—In general the descriptions given of the symptomatology of trypanosomiasis in various animals show a great similarity, though there is much variation in individual cases. The average clinical picture, according to Musgrave and Clegg, is as follows: After an incubation period which varies in the same class of animals and in those of different species, as well as with the conditions of infection, and during which the animal remains perfectly well, the first symptom to be noticed is a rise of temperature. For some days a remittent or intermittent fever may be the only evidence of illness. Later on the animal becomes somewhat stupid; watery, catarrhal discharges from the nose and eyes appear; the hair becomes roughened and falls out in places and the peripheral lymph nodes are enlarged. Finally, the catarrhal discharges become more profuse and the secretions more tenacious and even purulent; marked emaciation develops; edema of the genitals and dependent parts appears; a staggering gait, particularly of the hind parts, comes on, in some forms passing on to paralysis. This is followed by death. There may be various ecchymoses and skin eruptions. Parasites are found in the blood more or less regularly after the appearance of the fever. They are often more numerous in the enlarged lymph nodes and in the bloody edematous areas than in the general circulation.

The *autopsy* shows general anæmia, an enlarged spleen with hypertrophied follicles, more or less gelatinous material in the adipose tissue, the liver slightly enlarged, a small amount of serous exudate in serous cavities, edematous condition, and small hemorrhages in various tissues. There is a relative increase of the mononuclears in the blood.

The *duration* varies from a few days to many months. The *prognosis* seems to be influenced to a certain extent by the species of host. It is probably always fatal in horses. Some cattle recover. The chief cause of death is possibly a toxic substance, though no definite toxin has been isolated. Mechanical disturbances (emboli, etc.) also probably play a part in producing death.

**Man.**—*Sleeping sickness*, or *human trypanosomiasis*, is an endemic disease in certain regions of equatorial Africa. Neither age nor sex are predisposing factors, but occupation and social position seem to have a marked influence, the great majority of cases occurring among very poor field workers. As these workers are all negroes, the question of the relative influence of race cannot be determined. The white race, however, is not immune, as has been frequently shown.

*T. gambiense*, the trypanosome first shown to be pathogenic for human beings, is irregularly pathogenic for some monkeys (*Macacus rhesus* and others), for dogs, cats and rats. It is less pathogenic for mice, guinea-pigs, rabbits, horses, baboons, cattle, and swine. In fact it has a very wide range of pathogenicity.

Flies (*Gl. palpalis*) have been found to be infected with this trypanosome in areas which have had no human population for several years, a longer time than the life of a fly. And since trypanosomes are not known to be hereditarily transmitted, it is concluded that certain mammals are harboring the trypanosomes, thus acting as reservoirs.

**Symptoms.**—The course of the disease is very insidious, as the trypanosomes may exist in the blood for a long time before entering and growing in

the cerebrospinal fluid and causing the characteristic symptoms. Therefore the symptoms may be divided into two stages. In the first stage there is only an irregular fever with enlargement of the peripheral lymph nodes. In the second stage the fever becomes hectic, the pulse is constantly increased; there are neuralgic pains, partial edemas and erythemas, trembling of the muscles, gradually increasing weakness, emaciation, and lethargy. The somnolence increases until a comatose condition is developed and death occurs. In the second stage trypanosomes are always found in the cerebrospinal fluid. Throughout the disease they are usually found in small numbers in the blood.

**Duration.**—The first stage may last for several years; the second, from four to eight months. The percentage of deaths in cases reaching the second stage is 100. Whether some in the first stage recover is not yet certain.

**Pathological Changes.**—Congestion of the meninges; increased quantity of cerebrospinal fluid; hypertrophy of spleen, liver, and lymphatic ganglia; diminished hemoglobin and number of red cells; number of leukocytes about normal, but a relative increase of eosinophiles, mast cells, and lymphocytes. Enlargement of the superficial lymph nodes has been noted as an early symptom and has thus been made use of in diagnosis. Dutton and Todd found that 91 per cent. of natives in the Congo Free State, who had posterior cervical glands enlarged, showed trypanosomes in the punctured gland juice.

**Diagnosis of Trypanosomiasis in General.**—This should be made as early as possible in order to prevent the spread of the disease. An early positive diagnosis can only be made by the determination of the peripheral infection. This is done in two ways: first, by microscopic examination of freshly drawn blood, cerebrospinal fluid, or tissue from enlarged peripheral lymph nodes; second, by animal inoculation of the blood or other tissue. In the microscopic examination it may be necessary to examine the blood of the suspected animal for several days in succession. The parasites are rarely absent in the early stages in domestic animals for more than a few days at a time, while in man the time may be much longer.

**Methods of Examination.**—**BLOOD.**—If the direct examination of the blood is negative, 10 c.c. should be withdrawn from the vein, and after adding a tenth of its volume of citrate of sodium it should be centrifuged for ten minutes, and the sediment examined in hanging drop and in smear. The great majority of the parasites will be found collected with the white cells in the thin white layer between red blood cells and serum, which may easily be removed with a fine pipette. The parasites are readily detected with the low power of the microscope ( $\times 100$ ) by areas of irregularly moving cells. If only a small amount of blood can be obtained, the tiny tubes recommended by Wright in his opsonin work (p. 219) may be used.

**CEREBROSPINAL FLUID.**—Ten c.c. of the fluid withdrawn by lumbar puncture should be centrifuged for fifteen minutes, and the deposit should be examined under 150 to 200 diameter magnification.

*Cultures* sometimes give positive results, especially from infections in lower animals and in infections with *T. rhodesiense*.

**THE INOCULATION TEST.**—If the trypanosomes cannot be found by the above methods, animal inoculation should always be made. Monkeys, if possible, should be used, or if monkeys cannot be obtained, dogs or rats may be used. A few drops to 1 c.c. of the blood or other



tissue from the suspected animal should be inoculated intraperitoneally or subcutaneously.

Smears may be stained by any modification of the Romanowsky method. Giemsa's method (p. 82) gives good results (Plate IV, Figs. 1, 2).

**Prophylaxis.**—The disease is readily controlled by preventive measures. There should be strict quarantine regulations governing the importation of animals. When the disease has once appeared, the following general measures should be taken: (1) Suspected animals should be isolated. (2) All infected animals should be destroyed. (3) As far as possible, all biting insects should be destroyed, and the land in the neighborhood of human habitation should be suitably cultivated. (4) The bodies of infected animals should be protected from biting insects for at least twenty-four hours after death. (5) Susceptible animals should, if possible, be made immune. (6) All means possible should be used to exterminate the reservoirs. In sparsely settled districts the natives may be removed from the fly area.

**Treatment.**—The whole question of treatment is still in the experimental stage. The chronic course of the disease with relapses often after long intervals makes it impossible, especially in cases of human trypanosomiasis, to come quickly to a conclusion in regard to the efficiency of any drug. Many drugs have been found to possess trypanocidal properties to a certain extent.

Atoxyl (p-amino-phenyl-arsenic acid), introduced by Thomas (1905) and used first by Thomas and Breinl in treatment of experimental trypanosomiasis, proved to have a beneficial effect in the different forms of this disease.

The good reports received from the use of other arsenic compounds introduced by Ehrlich, namely salvarsan and neosalvarsan, have continued.

**Serum Therapy.**—Various normal sera from different animals have been tried with practically no success. A few have prolonged life. Thus Laveran and Mesnil state that human serum injected in sufficient quantities shows manifest action on the disease, and that sometimes cure results in mice and rats. Further, by alternating human serum with arsenic they obtained still better results. Kanthack, Durham, and Blandford showed that animals recovering from trypanosoma infection were immune to further infection. Rabinowitsch and Kempner have made a very careful study of immune serum produced by *T. lewisi*. They have shown that an animal may be hyperimmunized and that then its serum, in comparatively large doses, inoculated into mice at the same time as the trypanosomes, or twenty-four hours before or after, allows no development of the organisms. Laveran and Mesnil state that the serum causes the rapid destruction of the organisms by the leukocytes, though MacNeal, on the other hand, states that the trypanosomes are destroyed by a cytolytic action of the serum. This immune serum also has a similar action on the trypanosoma of dourine. The serum of animals hyperimmunized against other varieties of trypanosoma is not as active as that obtained by the inoculation of *T. lewisi*. Koch suggested that immunity might be produced by the inoculation of attenuated parasites, and Novy and MacNeal have

succeeded in attenuating cultures of *T. brucei*, and have obtained some success in protecting experimental animals against virulent culture.

**Complement-fixation.**—The earlier tests did not promise practical results. Recently, however, several investigators have given more favorable reports. In this country Mohler claims that the test is of great worth in diagnosing dourine, many cases of which have occurred in some of our western states (notably Iowa and Montana). Mohler, Eichhorn, Buck, and Traum state that they use a fresh antigen prepared from the spleens of rats dead after infection with surra (used because it is easier to transmit than is dourine). The antigen is simply a filtered emulsion in salt solution of the infected spleen. The emulsion from each spleen is made up to 40 c.c. by addition of salt solution. A fresh antigen must be prepared and titered (see Part I) each day. The results have been controlled by autopsies of animals giving a positive reaction.

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## CHAPTER XXXIX.

### SPIROCHETA AND ALLIES.

THE genus *Spirocheta* was introduced by Ehrenberg in 1838, who differentiated it from spirillum by its flexibility. Schaudinn, in 1905, thought he saw an undulating membrane in *Spirocheta refringens*, so he added this characteristic to the genus and considers that thus its relationship to the flagellated protozoa, genus *Trypanosoma*, is indicated.

Since the appearance of the work of Schaudinn and Hoffmann (1905) showing the etiological relationship of a spirochete to syphilis, the *Spirochetæ* have been brought into great prominence.

Numerous spirochetes and spiral organisms have been described, some associated with *Spirocheta pallida* (*Treponema pallidum*) in syphilis, some in other lesions or in the normal secretions of both man and the lower animals; and still the question as to their classification is unsettled. The majority of observers, however, are willing to admit that the structure of many of the varieties classed with this group is more complicated than that of bacteria and that hence the group may be an intermediate one between protozoa and bacteria.

The chief reasons given for considering spirochetes protozoa are: (1) the flexibility and the indications in many of longitudinal division and of undulating membrane; (2) the demonstration of forms intermediate between the trypanosomes and the spirochetes; (3) the spirochetal forms of certain trypanosomes (*T. noctuæ*); (4) stages of development in the louse and tick; (5) dissolution by certain chemicals such as saponin or sodium taurocholate in contrast to the resistance shown by most bacteria.

In favor of the bacterial nature of spirochetes are: (1) the rigidity of some forms, the lack of undulating membrane in most and of definite nuclear apparatus in all, and the evidence of transverse division in all and of flagella arising from the periplast in some; (2) the cultivation of certain forms (e. g., *Sp. refringens*, by Levaditi; *Sp. obermeieri* by Novy; several spirochetes by Noguchi and others) for many generations without development of trypanosome forms.

The classification then of the group of organisms commonly called spirochetes is still undecided. Noguchi in his recent Harvey lecture goes into details of the various groupings proposed. The first classification quoted is given here:

#### CLASSIFICATION AFTER GONDER. Spironemaceæ (Gross, 1910).

<i>Spirocheta</i> . . . . . (Ehrenberg, 1838)	Type: <i>Spirocheta plicatilis</i> ; all free living.
<i>Cristispira</i> . . . . . (Gross, 1910)	Type: <i>Cristispira balbianii</i> , and other varieties found in mussels.
<i>Spironema</i> . . . . . (Vicillemin, 1905)	Type: <i>Spironema recurrentis</i> , and other parasitic varieties living in blood.
<i>Treponema</i> . . . . . (Schaudinn, 1905)	Type: <i>Treponema pallidum</i> , and other varieties with closely set spirals.

It may be well to note briefly the chief characteristics of the more familiar non-pathogenic species in order better to understand the relationships between them and the *Treponema pallidum* and other pathogenic forms.

**Material and Methods for Study.**—A large spiral organism (*Cristispira balbianii*) closely related to the spirochetes is found in the stomach of oysters fresh from salt water. Smaller spirochetes are frequently found in human mouths. When fresh syphilitic or relapsing fever material can be obtained this should be examined. The *Treponema pallidum* (the spiral organism of syphilis), because of its low refractive index, is seen when alive with difficulty by the ordinary microscope, but with the dark-stage illumination, especially if a drop of distilled water is added to the serum containing the organisms, it is seen distinctly and its motion and structure may be more easily studied. The fluid containing the organisms should be dropped on an ordinary glass slide, covered with a thin cover-glass, and well sealed with vaselin, as most spirochetes are anaërobic. Material may be obtained from syphilitic lesions as follows: The lesion is first thoroughly washed and dried with distilled water and sterile gauze. Part of the base and margin is then scraped with a curette until the superficial tissue is removed and blood appears. The blood is wiped away with sterile gauze until clear serum begins to ooze. A drop of this serum is used for examination.

Smears should be made as thin as possible and may be stained (1) by Giemsa according to the method on page 82 (*Tr. pallidum* stains reddish). A modification of Giemsa, used by Schereschewsky, has been highly recommended by various workers; (2) by Goldhorn's method (p. 83).

**Cultures.**—Pure cultures have been obtained of the *Treponema dentium* in the following manner: Poured serum-agar plates are made of various dilutions of material from the mouth containing these spirochetes. After being kept in the thermostat at 37° C. under anaërobic conditions for nine to twelve days the spirochetel colonies are fished and planted in agar tubes as stick cultures.

Pure cultures of *Spironema recurrentis* (*Spirocheta obermeieri*) by Novy and of *Spironema refringens* by Levaditi have been obtained by growing in collodion sacs. (For other culture experiments see below.)

**Cristispira Balbianii (Certes).**—This immense form, next largest known to the *Spirocheta plicatilis* Ehrenberg, may be found in the oyster's crystalline style. It is important for study because it is apparently a transitional form. In fact it is considered a trypanosome by Perrin and others. Mühlens gives its characteristics as follows: Length  $26\mu$  to  $120\mu$ , in width  $\frac{1}{2}\mu$  to  $3\mu$ . The body is flattened and possesses a ridge, or crista, which is visible during life on some individuals. It has 4 to 8 flat, wide spiral coils. Its movements are lively, similar to those of trypanosomes, but more corkscrew-like. During motion its form is apparently easily changed. The rim of the membrane-like ridge does not end in a free flagellum, but one end of it seems to be attached to a triangular mass of chromatin (basal granule, blepharoplast?) which is a part of the central chromatin material. The nuclear material is arranged in a more or less spiral band along the entire centre of the organism.

Before division this nuclear band, after passing through chromosome-like changes, breaks up into pairs, and division takes place longitudinally between them. Division is often incomplete for a time, the two ends remaining attached.

**Spironema Balanitidis.**—This is a spirochete found by Simon in *Balanitis circinata* and regarded by some as the specific cause of this disease. Hoffmann and Prowazek describe it as a rather strongly refractive, actively motile, band-shaped organism, shorter and thicker than *Treponema pallidum*, with 6 to 10 coils staining bluish red with Giemsa's method and at either end a periplastic cilium. No definite undulating membrane has been demonstrated.

Mühlens thinks this may be identical with *Spironema refringens*. Levaditi has reported cultivating it (see below under *Treponema Pallidum*).

**The Mouth Spirochetes.**—Several species of non-pathogenic forms are commonly found in normal mouths.

1. *Spiroonema Buccalis* (Cohn).—Length,  $10\mu$  to  $20\mu$ ; thickness,  $\frac{1}{2}\mu$  to  $\frac{3}{4}\mu$ . It has 3 to 10 irregular flat coils. No true cilia have been demonstrated. The claim of Schaudinn, Hoffmann and Prowazek that it has an undulating membrane has not been corroborated. It stains violet with Giemsa.

2. *Treponema Dentium* (Koch).—This is much smaller than the previous form. It is as thin as the pallidum and is somewhat similar to it in refraction, staining qualities, and in the fixity of its coils during motion. It is somewhat smaller than the pallidum and stains a little more easily with Löffler's flagella stain, and flagella have been demonstrated. Neither definite undulating membrane nor nuclear material has been seen. It is  $4\mu$  to  $12\mu$  long, and has 4 to 20 regular spirals of about the same appearance as those of the pallidum. Pure cultures have been made from this spirochete as described above. Several varieties have been described.

3. *Treponema macrodentium*, a form in appearance between these two has been found in the mouth. This also is somewhat similar to the pallidum, but it is larger and has less regular spirals; moreover, it stains more intensely with the blue of Giemsa, only in poorly prepared specimens does it appear red.

**Spiroonema Refringens.**—*Spiroonema refringens* is also found in the mouth, but it is especially interesting from the fact that it is so often found associated with the *Treponema pallidum* in the various lesions of syphilis. It is not in such large numbers as the pallidum and probably bears the relation of a restricted secondary invader. It is generally longer than the pallidum ( $10\mu$  to  $30\mu$ ) and much thicker ( $\frac{1}{2}\mu$  to  $\frac{3}{4}\mu$ ). In life it is much more refractive. It has 3 to 15 irregular wide, flat spirals which change their shape during motion. Its movements are much more lively than those of pallidum. With Giemsa it stains quickly and easily, a blue to a blue-violet tone, according to the length of staining. Schaudinn states that it possesses an undulating membrane. Levaditi claims to have demonstrated terminal cilia for this organism and to have cultivated it in collodion sacs in the rabbit's peritoneum.

**Spiroonema Vincenti.**—Accompanying the fusiform bacilli in Vincent's angina (see p. 450) are many spirochetes similar to the "middle form" found in the mouth. Whether they are identical with these spirochetes or whether they are a special variety (or, as some think, a second form of the fusiform bacillus) still remains to be determined. Their relationship to the disease is also uncertain.

**Spirochetes in Tumors.**—Löwenthal, Borrel, and others found spirochetes in small numbers in certain mouse tumors. Ewing and Beebe found a few in some dog tumors and others have reported their occasional presence in both ulcerating and non-ulcerating human tumors, but apparently never in sufficient numbers to account for the tissue reaction. Gaylord, however, found that in repeated transplants of a mouse tumor, as the inoculated material became more virulent the number of spirochetes greatly increased. Calkins studied the morphology of Gaylord's spirochete and decided it to be a distinct species. He has also found this species in primary as well as in transplanted tumors. It is much shorter and thicker than the pallidum, and has blunt ends. It closely resembles the spirochetes found comparatively frequently by Tyzzer and others in apparently normal mice, though the possibility of infection in these cases was not ruled out.

**Miscellaneous Spirochetæ.**—Besides the spirochetes found in syphilis, in frambesia, and the spiral organisms causing African and European relapsing fevers, all of which will be described below, spirochetes have been found (1) in the normal intestinal tract of mosquitoes and human beings as well as in the diarrheal stools of the latter; (2) in the blood of mice and fowls (*S. gallinarum* causing relapsing fever in fowls and *Sp. anserina* found in similar condition of geese); (3) in various ulcerative and gangrenous process of man.

The fowl spirochetes have been most studied. Marchoux and Salimbeni were the first to show that the tick, *Argas persicus*, is a carrier of these spirochetes.

The mechanism whereby the tick infects the fowl has been minutely worked out by Nuttall and his associates.

Noguchi has cultivated several species of spirochetes by the same method he used for growing *Tr. pallidum* (see below). Some of these he claims as new species, namely *Tr. macrodentium* and *Tr. microdentium* from the mouth, *S. phagedensis* from human genitals, *Tr. calligyrum* from condylomata and *T. mucosum* from pyorrhea alveolaris. Noguchi also reports the cultivation in successive transplants of *Sp. recurrentis* and *Sp. duttoni*.

King and Bauslack report finding a spirochete (*S. hyos*) in the blood of pigs suffering from hog cholera, which they consider the cause of the disease.

### TREPONEMA PALLIDUM (SPIROCHETA PALLIDA).

This organism is found in large numbers in *syphilis*, an infectious disease of human beings, characterized by its long course and by the definite stages of its clinical manifestations.

**Historical Note.**—Notwithstanding the fact that syphilis is one of the oldest diseases known and studied, only comparatively recently has definite light been thrown upon its cause in the discovery of the *Treponema pallidum* (Schaudinn and Hoffmann, 1905).

Before this it was thought that the bacillus described by Lustgarten (1884) and others as occurring in small numbers in the lesions of syphilis bore an etiological relationship to the disease, but there were no evidences to support this view. Many other bacteria have been erroneously regarded as the probable cause of syphilis.

From time to time various observers have described protozoan-like bodies in syphilitic lesions, but their observations have not been confirmed.

Schaudinn announced early in 1905 that working with Hoffmann he found in the fresh exudates of chancre a spiral organism possessing characteristics similar to those of the spirochetes and he named it *Spirocheta pallida*. Later he concluded that this organism was individual enough (that is, it showed no undulating membrane, but possessed flagella) to be placed in a separate genus, so he called it *Treponema pallidum*. He thought that the organism was the cause of the disease. Since then there have been extensive studies on human syphilis and on experimental syphilis in lower animals with the result that the work of Schaudinn and Hoffmann has been abundantly corroborated and many new facts, given below, have been brought out.

**The Organism** (Fig. 174).—The *Treponema pallidum* is a very delicate structure closely resembling in morphology and staining reactions the *Treponema dentium*. It is somewhat longer,  $4\mu$  to  $20\mu$  long (average  $10\mu$ ), and thinner,  $\frac{1}{2}\mu$  to  $\frac{1}{4}\mu$  in diameter. It has three to twenty sharp, deep spirals. The relationship between the length and the depth of the spirals is different in the two species; in *Treponema pallidum* length is to depth as 1 is to 1–1.5 ( $1\mu$  long and  $1\mu$  to  $1.5\mu$  deep), while in *Treponema dentium* the average relationship is 1 to 0.5, the spirals being more shallow. The angle of the spiral turn is very sharp in both forms (more than  $90^\circ$ ).

Flagella-like anterior and posterior prolongations are often seen in the *pallidum*. The double flagella occurring rarely at one end are interpreted by Schaudinn as beginning longitudinal division. Schaudinn states that the division occurs very quickly (hence the reason why so few dividing forms are seen in stained preparations) and that it may be followed only by the most experienced observers during life.

In the living condition the organism is not very refractive, so it is seen at first with difficulty. Its characteristic movements are rotation on its long axis, quivering movements up and down the spiral which is comparatively rigid, slight forward and backward motion and bending of the entire body. By the use of the ultramicroscope the motility of the organism is clearly seen (Fig. 173).



FIG. 173.—The two spirochetes in the centre are *Tr. pallidum*; the three others, *Sp. refringens*. (Schaudinn and Hoffmann.)



FIG. 174.—*Treponema pallidum* appearing as bright refractive body on a dark field as shown by India ink or ultramicroscope.

**Examination in Fixed Preparations.**—The staining methods that have given the most satisfactory results may be found on page 83. The spirochetes on the whole take all stains faintly, but they may be clearly demonstrated in smears by the India ink method (Fig. 174) and in sections by the silver impregnation method.

**Cultivation.**—In 1909 Levaditi and McIntosh obtained impure cultures of spirochetes in collodion sacs containing human serum and syphilitic material and placed in the peritoneal cavity of a monkey (*Macacus cynomolys*). Schereschewsky reported that he had obtained impure cultures of a spirochete from syphilitic lesions and blood in the following culture medium: horse serum sterilized by heat (58° to 60° C.) until it is of jelly-like consistency, and afterward autolyzed at 37° for three days. A piece of tissue excised from the lesion (*e. g.*, base of a papule or part of a lymph node) is inoculated into this medium, and grown at 37° C. The culture begins in three days, but the optimum is reached in five to twelve days.

Mühlens (1909) and later Hoffmann (1911), reported that they had also obtained cultures of a pallidum-like spirochete from syphilitic lymph nodes, grown at first in Schereschewsky's medium and afterward

transplanted to broth and grown anaerobically. Animal experiments are negative.

Levaditi and Stanesco about the same time reported growing two species of spirochetes from a case of balanitis. One, a new one, which they found very like pallidum, but non-pathogenic for monkeys, and which they named *S. gracilis*; the other *S. balanitidis*. They employed as media (1) collodion sacs in tubes of fluid horse serum; (2) horse or human serum heated to 75° C. These spirochetes were never obtained in pure culture.

Noguchi (1911) obtained pure cultures from syphilitic lesions in the rabbit, and later from human beings. His culture medium contained in deep tubes was a mixture of 1 part of ascitic or hydrocele fluid and 2 parts of 2 per cent. agar, in which was placed a small portion of sterile rabbit's kidney or other organ. The medium was covered by a deep layer of alboline. The spirochetes inoculated along a central stick grow out into the medium as a diffuse layer after ten days at 33.5° C., while most contaminating bacteria remain in the line of inoculation. Subcultures made away from the contaminating growths, finally become pure. Noguchi states that he has obtained syphilitic papules by scarifying monkeys (*cercopithecus* and *macacus*), and that such monkeys' blood gives a positive Wassermann reaction.

Zinsser and Hopkins as well as others have corroborated this work of Noguchi. Gilbert found that later pure cultures of spirochetes grow abundantly when slant egg media are used instead of kidney tissue.

**Pathogenesis.**—So far as is known, syphilis spontaneously appears only in man. Since 1879, when Klebs stated that he had produced syphilis in monkeys by the inoculation of human virus, various experimenters have reported its transmissibility to these animals by direct inoculation. Most of the earlier reports did not state the exact identity of the animals employed nor did they give details of methods and results.

Metchnikoff and Roux, in 1903, produced a typical chancre on the genital mucosa of the young chimpanzee twenty-six days after inoculation. The essential lesion was followed by inguinal adenitis, and thirty days later by a generalized papular eruption. The virus was transferred in this case to lower monkeys. Most monkeys developed a primary lesion only, but some had abundant secondaries.

Since the discovery of the *Tr. pallidum*, experiments on monkeys have been more numerous and have been followed by more helpful results. More has been learned about the course of the infection in man, the evidence in favor of the *Tr. pallidum* being the cause of the disease has been strengthened, and many interesting investigations in regard to immunity have been made. Usually inoculations made by scarification on eyebrows or genitals are successful. The primary lesions are seen generally about thirty days after infection. In somewhat more than half the cases, after a slightly longer period, secondary symptoms (squamous papules on skin, and mucous patches in mouth) show themselves. No tertiary symptoms have been observed. Rabbits were first successfully inoculated by Hansell, then by Bertarelli. The eye and the testes are favorable localities. Generalized syphilis has been produced in young rabbits by intracardiac inoculation (Uhlenhuth and Mulzer).

Important features in regard to course of the infection have been summarized by Ewing as follows: "If the virus is applied to the broken epithelium,



a chancre develops, but if similar virus is inoculated into the subcutaneous tissue an initial lesion does not follow, immunity does not develop, and the animals remain susceptible to subsequent inoculation of the epithelium. Yet in several instances Neisser was unable to produce chancres in monkeys which had previously received subcutaneous injections of syphilitic material, indicating that immunity may sometimes appear after such subcutaneous injections. Possibly the leukocytes of the subcutaneous tissue destroy the virus before it can begin to multiply. Hence small superficial wounds may be more dangerous in man than deep ones. Nevertheless, it is recorded by Jullien that two French surgeons, accidentally inoculated by deep needle punctures, developed pronounced signs of constitutional syphilis, as attested by Fournier, but failed at any time to show signs of a chancre at the point of inoculation.

"In monkeys the virus exhibits a certain choice of epithelium for its entry. The abdominal skin resists the entry, the eyebrows and genitals are most readily inoculable in apes, and the palpebral borders in catharinians. The period of incubation varies from thirty days, on the average, in the chimpanzee, to twenty-three days in lower monkeys, but the shorter the incubation, the shorter and less severe the subsequent disease.

"That the virus circulates in the blood in certain stages of syphilis has been clearly shown experimentally. Although Neisser inoculated human subjects with the blood of florid syphilis without effect, a result which is now explicable. Hoffmann, in two of four experiments, produced syphilis in monkeys (*Macacus rhesus*) by inoculating the skin with human blood drawn forty days and six months after the appearance of the chancre. The resulting primary lesions were typical, appearing after the usual incubation and showing a characteristic histological structure and the presence of *Tr. pallidum*.

"Syphilographers are agreed that tertiary lesions are not contagious. Experimental studies have shown, however, that some tertiary lesions are capable of transmitting the disease. Salmon had negative results with an ulcerated gumma in the eighth year of the disease. Yet Neisser produced chancres and secondaries in a gibbon and in a macacus with the material from a non-ulcerated gumma (duration unknown), but the periods of incubation were very long, fifty-one and sixty-eight days. None of these observations invalidates the clinical experience that tertiary lesions are practically harmless for the patient's neighbors, but suggest greater caution in dealing with tertiary lesions.

"According to Colles's law, a mother who gives birth to a syphilitic infant may not herself contract the disease, but thereafter remains immune to inoculation. This law may be explained by the infection of the embryo or ovum, and the transference of immunity to the mother by the blood or by some other method. The probable mode of origin of the maternal immunity is suggested by an observation of Buschke and Fischer who found spirochetes in the inguinal lymph nodes of such a case which remained entirely free from the symptoms of the disease. Levaditi and Sauvage claim to have shown that *Tr. pallidum* is capable of invading the ovum. Finger and Landsteiner found the semen in one case of secondary lues, infectious for apes, but in other cases their results were negative. It is therefore only necessary to suppose an occasional escape from the genital tract in order to complete the necessary conditions for the infection of the embryo with immunity in the mother.

"Neisser endeavored to determine the degree and duration of the infectivity of the organs of monkeys and found that the virus persists especially in the blood-forming organs, spleen, lymph nodes, and marrow, while in the testicle also the virus is long preserved in active form. The other organs gave entirely negative results."

The virus is not filtrable, though the spirochetes can grow through the pores of Berkefeld filter V and N in about four days (Noguchi). It is readily destroyed by heat (52°C.).

*Luetin*.—Noguchi found that an extract from his cultures, which he calls luetin, gives a characteristic cutaneous reaction in syphilitic infection. In normal

persons there is a slight local erythema, with possibly a small papule on the second day. In syphilitics there are three types of positive reaction:

1. Papular type. Large indurated red papule which increases for about four days.

2. Pustular type. The papule changes first into a vesicle and then into a pustule.

3. Torpid type. After a ten-day latent period a small papule forms which changes into a pustule.

These observations of Noguchi have been corroborated by Cohan and Robinson.

**Symptoms in Man.**—The course of the disease is divided into three stages: primary, secondary, and tertiary. The general character of the lesions in these stages is a more or less circumscribed formation of new tissue which is largely made up of small spheroidal cells alone or accompanied by a few endothelioid cells, and occasional giant cells.

The initial or primary lesion occurs in the form of a papule which develops into the so-called chancre, an ulcer with hardened base. Following this there is hyperplasia of the nearest lymph nodes. These lesions subside and six or seven weeks later the secondary lesions appear in various general eruptions on skin and mucous membranes and in other constitutional disturbances. The tertiary lesions which consist principally of the masses of new tissue called gummata are found throughout the viscera and in the periosteum.

Schaudinn's spirochetes have been demonstrated in all the lesions of syphilis (they are most easily demonstrated in the primary and secondary lesions), including the congenital types.

**Immunity.**—Natural immunity in syphilis is very peculiar. After the development of the primary lesions, man is usually insusceptible to reinoculation during the active stage of the disease, but during all stages both man and monkey can, in some cases, be reinoculated. Reinoculation in the tertiary stage gives precocious lesions of the tertiary type, gummata, and tubercles. Neisser found reinoculation from twenty-four to one hundred and four days after primary inoculation in monkeys sometimes effective, more often negative. During the stage when the skin is refractory to inoculation secondaries develop, showing that there is no complete immunity of the skin to the virus, since the *Treponema* is abundantly present in the lesions. Neisser suggests that cutaneous secondaries develop at periods of relative deficiency of immunity. He has shown that failure of reinoculation is not due to immunity to foreign infection and susceptibility to auto-infection, since the patient's own virus in both man and monkey is ineffective.

Many attempts to produce active immunity artificially have failed. Zinsser, Hopkins and McBurney state that they can obtain from rabbits which have been inoculated with repeated doses of these culture spirochetes, sera with antibodies (agglutinin) for their non-virulent cultures of *Tr. pallidum* but not for the recently isolated virulent strains.

**Passive Immunization.**—Injection of large quantities of serum of syphilitics into chimpanzees has failed to produce definite immunity, neither has the serum of animals repeatedly inoculated with syphilitic

virus or with pure cultures of the pallidum had any prophylactic effect against the virus.

**The Wassermann Reaction.**—Wassermann, Neisser, and Bruck were the first to apply the Bordet-Gengou phenomenon (see p. 188) to the diagnosis of syphilis. According to most workers, enough work has been done since then to establish its value as a diagnostic test. Some interesting points have been brought out in connection with this study, and many different methods have been recommended.

This test has been very widely used and positive results have been obtained in an immense number of cases (over 90 per cent. of cases with active general infection). In general paralysis and in the majority of cases of tabes a positive reaction occurs which may be given also by the cerebrospinal fluid. On account of the difficult technic involved it can only be of use in the hands of experienced workers. Positive results have been reported in a number of other diseases as well as syphilis, but in many cases these results have not been generally accepted, and in other cases the diseases showing positive reaction as yaws, leprosy, dourine, etc., have been, as a rule, confined to the tropical countries, or else the positive reaction has been found only during a limited stage of the disease, as in scarlet fever, or the differential diagnosis is otherwise marked, as with tuberculosis. The general opinion at the present time is that the test is of great practical value in the majority of cases of syphilis. It is used as a routine public health measure in New York City and several other centres. The reaction is practically always present during the secondary stage of the disease. The reaction gradually disappears when the disease becomes inactive or is cured. It may disappear before a cure is established, to reappear if an active process starts. Mercury treatment diminishes or annuls the reaction with an occasional intensification after a few treatments; while treatment with salvarsan may at first intensify it or make a negative reaction positive. A good recent report of the diagnosis and treatment of syphilis is given by Fordyce.

The nature of the reaction and the technic of the test are considered in Part I, p. 196.

**Colloidal Gold Test.**—In 1912 Lange applied to spinal fluids the principle brought out by Zsigmondy (1901) that solutions of either electrolytes or of proteins precipitate colloidal gold, but that when both electrolytes and proteins are present the gold is not precipitated. Miller and his co-workers have been able to standardize the reagent, so the test has become more reliable.

The test is simply another aid in helping to detect certain central nervous system involvements not responding so specifically to other tests. The test consists of a series of color changes in graded dilutions of spinal fluid, varying from negative salmon-red, then red-blue, blue, blue-gray, to colorless. Miller has worked out a "paretic curve."

**Spirochetes in Frambesia Tropica (Yaws).**—Castellani, in 1906, announced that he had found in yaws a spiral organism which is called *Treponema pertenue*. He determined that monkeys (*macacus*,

*semnopithecus*) are susceptible to inoculations with material from yaws patients apparently containing only this spirochete. Such material filtered is inert. He states that monkeys successfully inoculated with yaws do not become immune for syphilis, neither do those having had syphilis become immune for yaws. Castellani further states that specific characteristics between the two diseases are also brought out by means of the Wassermann reaction. His work has been corroborated by several observers. Frambesia lesions similar to those produced by syphilis in the testicles of rabbits have been obtained by Nichols. Levaditi and Nallan-Larrier state that monkeys infected with syphilis are refractory to yaws, while those infected with yaws are susceptible to syphilis; therefore they conclude that yaws is a mild form of syphilis.

**Spirochetes in Infectious Jaundice.**—Recently Inada, Ido and their collaborators in Japan, and Heubner and Reiter in Germany have reported the presence of spirochetes (*S. icterohemorrhagia*) in infectious jaundice or Weil's disease. This work was confirmed by Stokes and by Noguchi. Ido and his co-workers claim to have found the same species in both house and field rats, and to have obtained active immunity in guinea-pigs against the organism.

**Spirochetes in Rat-bite Fever.**—Futaki and his associates have reported the presence of a spirochete similar to *S. recurrentis* in cases of rat-bite fever in Japan.

#### SPIRONEMA OBERMEIERI (*S. RECURRENTIS*) AND ALLIES.

The organisms in this group are classed with the spirochetes as protozoa by Schaudinn, Hartmann, Mühlens, and others, but by Norris, Novy, and others they are still placed with the bacteria. Novy and Knapp have made extensive studies of *S. obermeieri* (the cause of relapsing fever in Europe) as well as of *S. duttoni* (the cause of tick fever), spirochetes from American relapsing fever, and *S. gallinarum* (fowl spirochete) and consider that they have demonstrated their bacterial nature and that many, if not all, spirochetes should be placed in this group.

**Spironema (spirillum) obermeieri** was first observed by Obermeier in 1873 in the blood of persons suffering from relapsing fever. It was found in large numbers during the height of the fever, it disappeared about the time of the crisis, and reappeared during the relapses. It was not found in other diseases. Obermeier considered it the cause of the disease, and his views were shown to be correct by the production of the disease in man and ape through experimental inoculation.

**Morphology.**—The organisms are long, slender, flexible, spiral or wavy filaments, with pointed ends, from  $16\mu$  to  $40\mu$  in length and from one-quarter to one-third the thickness of the cholera spirillum ( $\frac{1}{2}\mu$  to  $\frac{3}{4}\mu$ ). Novy has demonstrated a terminal flagellum (Fig. 175). They possess three to twelve wide, more or less irregular spirals. They stain somewhat faintly with watery solutions of the basic aniline dyes, better with Löffler's or Kühne's methylene-blue solutions, or with carbol-

fuchsin; best with the Romanowsky method or its modifications. They are negative to Gram.

**Biological Characters.**—In fresh preparations from the blood the spirochetes exhibit active progressive movements, accompanied by very rapid rotation in the long axis of the spiral filaments or by undulating movements. They are found only in the blood or blood organs, never in the secretions, and only during the fever, not in the intermissions, or at most singly at the beginning of, or for a short time after, an attack.

When kept in blood serum, or a 0.6 per cent. solution of sodium chloride, they continue to exhibit active movements for a considerable time. They may be preserved alive and active for many days in sealed tubes. They are killed quickly at 60° C., but they remain alive for some time at 0° C. Unsuccessful efforts to cultivate them in artificial culture media have been made from time to time. Koch has observed



FIG. 175.—Photograph of *Sp. obermeieri* showing terminal flagellum.  $\times 3000$  diameters. (After Novy.)



FIG. 176.—*Spirocheta obermeieri* blood smear. Fuchsin.  $\times 1000$  diameters. (From Itzerott and Niemann.)

an increase in the length of the spirilla and the formation of a tangled mass of filaments. Novy finally succeeded in cultivating them in celloidin capsules placed in the peritoneum of rats. His culture remained virulent (with a slight loss) for many generations. Noguchi has also cultured them.

**Pathogenesis.**—In man, whether the disease is acquired naturally or by artificial inoculation, the organism causes the following symptoms: After a short period of incubation the temperature rises rapidly, remains high for five to seven days, and then returns to normal by crisis. About seven days later there is another sudden rise of temperature, but this time the crisis occurs sooner. A second or third relapse may occur. The organisms increase in numbers rapidly in the blood from the beginning of the fever, large numbers often being found in every microscopic field. They begin to disappear a short time before the crisis, and immediately after the crisis it is practically impossible to find them in the circulating blood. The mortality varies in different epi-

demics from 2 to 10 per cent. When monkeys are inoculated with human blood containing the spirilla, they become sick about three and a half days later, but show only the initial febrile attack or, at the most, an occasional short relapse. The organisms are found to have the same relation to the pyrexial periods as in man. Blood from one animal taken during the fever induces a similar febrile paroxysm when inoculated into another animal.

Metchnikoff showed that during the intermissions when the spirochetes disappeared from the circulating blood they accumulated in the spleen and were ingested in large numbers by certain phagocytes and finally were destroyed.

According to Lamb, a certain amount of immunity is conferred upon monkeys (*Macacus radiatus*) soon after an attack, but it disappears quickly. If the serum is removed during this time it is found to have some protective action when mixed with the blood containing spirilla, and also to cause agglutination of the organisms. Novy (1906) showed that a powerful specific germicidal body exists in the blood of rats during and after recovery, notably in the blood of hyperimmunized rats. An immunizing body probably distinct from this is also present. He also showed that passive immunity can be imparted by injections of recovered or hyperimmunized blood, that both active and passive immunity may last for months, and that the serum has both a preventive and a curative action. Agglutinins are also present in such a serum.

Breinl has shown that the immunity produced by *S. obermeieri* does not protect against *S. duttoni*, and *vice versa*. Neither does it protect against the Asiatic or American variety of this type of spirochete.

The strain found in Bombay seems to be more virulent than that in Europe.

**Spironema Duttoni.**—The organism shown by Dutton (1905) to be the cause of African tick fever is very similar morphologically to *S. obermeieri*, but Novy, Fränkel, and others have shown slight differences which make them believe that it is another variety, if not another species of this group.

**Spironema Carteri.**—This spirochete was described by Carter in 1877 as causing relapsing fever in Bombay. Monkeys were inoculated by Carter successfully with the human blood containing this spirochete.

**Spirochetes from Relapsing Fever in America.**—Recently Darling has reported a study of the relapsing fever in Panama. He isolated the organisms in two cases and studied their characteristics. He finds they agree with those reported by Carlisle, Norris, and Novy for the organisms isolated by Norris, but they can only be differentiated from the other relapsing fever spirochetes by animal inoculations and by the disease in humans. Moreover, he finds that in all probability a polyvalent serum may be necessary for cure, since the serum from one strain did not protect against the other strain.

**Filtrability of Spironema.**—A number of workers have demonstrated that spironema may pass through Berkefeld's N and V under pressure.

## INSECT CARRIERS OF SPIROCHETES AND ALLIES.

Each species of spirochete is conveyed by the bite of a specific arthropod. Many studies have been made on this subject since the work of Marchoux and Salimbeni on fowl spirochetes, and of Dutton and Todd on African tick fever. Nuttall and his associates have added much to our knowledge of ticks and other arthropods as vectors. Dutton and Todd demonstrated that *S. duttoni* can be transferred to monkeys by the bites of young ticks (*Ornithodoros moubata*) at their first feed after hatching from infected parents. He accidentally demonstrated the fact that the disease can be inoculated into human beings through a cut surface, for after a wound received at autopsy he developed the disease which eventually caused his death.

Koch corroborated these authors' results. Leishman demonstrated that the second generation of ticks may also infect. He was unable to find spirochetes in tissues of ticks later than ten days after feeding, though young ticks from eggs hatched after this time were infective. He found, however, granules which he considers changed spirochetes. Hindle agrees with him. The fact that ticks may be infective long after the disappearance of typical spirochetes, if they are placed at 30° to 35° C. is evidence of the infectious nature of these granules. Möller found that a tick may remain infective for a year and a half or more after its initial feed from an infected host. Möller also showed that the third generation of ticks might inherit infection. Sargeant and Foley and later Balfour have observed another type of relapsing fever in Africa, which they consider due to a different variety of spirochete (*S. berbera*). Mackie obtained strong evidence that the body louse is the chief carrier of *S. recurrentis*. His observations have been corroborated by a number of observers.

Nicolle and his colleagues found after twenty-four hours spirochetes disappear from gut of the louse (*P. vestimenti*) to reappear in from eight to twelve days and continue for eleven days and possibly longer. Monkeys inoculated with the contents of lice fifteen days after feeding develop relapsing fever.

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## CHAPTER XL.

### BODO. POLYMASTIGIDA.

#### **BODO LACERTÆ (GRASSI).**

*Bodo lacertæ* is frequently found in the intestinal contents of most of the higher animals, hence it is easily obtained for class study. A species of the *Bodo* has been observed in human urine (*Bodo urinarius*), but it is probably a harmless invader.

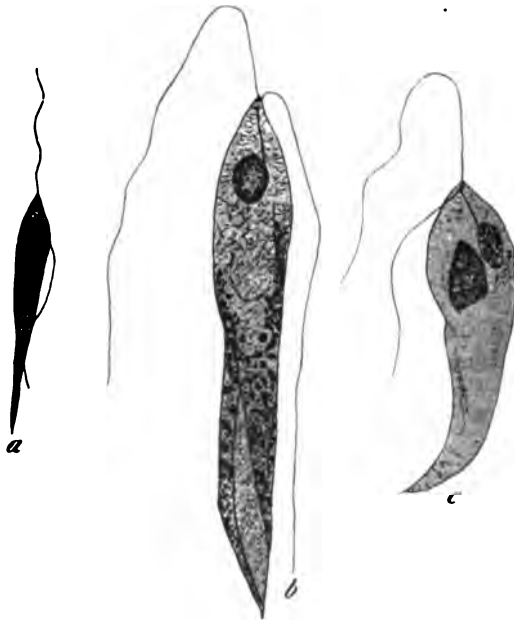


FIG. 177.—*Bodo lacertæ* (Grassi): *a*, living; *b* and *c* stained; *b* type without, *c* type with chromidium (1000 : 1). (After v. Prowazek, from Kisskalt and Hartmann.)

*Bodo lacertæ* is wedge-shaped, the posterior part of the body being turned a half to a whole spiral on itself. It possesses two characteristic flagella, equal in thickness but unequal in length. In motion the longer one is directed forward, while the shorter is carried backward, functioning as a rudder, or a towing flagellum. Both flagella spring from a basal granule which are well demonstrated by the iron hematoxylin stain. They are situated in the extreme anterior part of the body and are attached to the nucleus by a delicate fibril (Fig. 177, *b*, *c*, and Plate IV, Figs. 1, 3). The movement of the organism is character-

istic, it consists in a rapid irregular swimming in various directions with the anterior flagellum moving from side to side. The body itself shows a slightly sinuous motion.

There are two types of nuclei seen. First, the typical vesicular nucleus most frequently seen among the flagellates. This is round and has a definite membrane about which chromatin is arranged in irregular masses. In the centre, or eccentrically placed, is a compact karyosome. Iron hematoxylin preparations bring out an achromatic network between the chromatin masses and the karyosome. In the living condition the nucleus appears as a greenish glistening refractive vesicle. The second type of nuclear apparatus is seen in smaller organisms. This is a similar nucleus except that it is smaller and more compact; posterior to this is another nuclear-like body, varying much in shape and arrangement of chromatin (Fig. 177, c). This is the sexual chromidia.

The cytoplasm appears in iron hematoxylin stained specimens as finely reticular. It contains many deeply stained granules. There is no mouth opening. Food is taken in by osmosis.

In propagation, the two types just described develop differently. The first or ordinary type forms around division cysts. The flagella disappear and a delicate cyst membrane is formed. The increase in the size of the nucleus and the subsequent division may be followed in life. It lasts about twenty minutes. After a single or, more seldom, a double division of the cell, the daughter cells, while still within the cyst, form their flagella, become very motile, finally break the cyst wall and swim out. The second type reproduces, in the free living condition by longitudinal division. The basal granules divide, the principal nucleus divides by mitotic division, the chromidia by amitosis. The whole process can be seen in hematoxylin preparations. Sexual division in this species occurs in cysts by autogamy. It is not easily followed in life because of the high refraction of the cyst. The changes must therefore be studied in specimens stained with iron hematoxylin.

They are briefly as follows: The nucleus becomes larger and about its membrane appear small spheres of chromatin which finally leave the nucleus and gather together, forming the so-called chromidial or sexual nucleus, while the original or somatic nucleus gradually degenerates. The new nucleus divides amitotically into two daughter nuclei; from these two, smaller parts are then separated, as reduction nuclei, which also degenerate. The remaining parts of the two nuclei increase in size and then fuse to form a new nucleus. The organism may then leave the cyst or the cyst may become a lasting cyst and serve to infect a new host.

Besides this method of fructification by autogamy in a cyst, is seen, though seldom, a copulation between two individuals of different sizes which afterward become encysted and divide into two to sixteen daughter flagellates.

## POLYMASTIGIDA.

The order polymastigida consists of flagellates having several flagella projecting from different parts of the body. The majority of the forms known are parasitic in certain fish.

**Trichomonas Vaginalis.**—Donné, in 1837, described a form which he found in the human vagina, and which he therefore called *Trichomonas vaginalis*. It has been found by other observers to be a frequent habitant of the vagina at all ages. It is also found occasionally in the acid urine of both sexes. The mode of infection of the female is unknown. The body of the parasite at rest is pear-shaped, but during action its ameboid movements cause it to assume various shapes. The size varies from  $12\mu$  to  $25\mu$  long and  $8\mu$  to  $15\mu$  wide. The protoplasm is

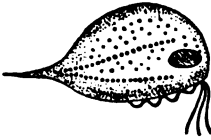


FIG. 178.—*Trichomonas vaginalis*.  
(Blochmann.)

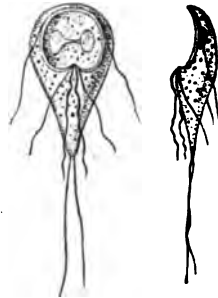


FIG. 179.—*Lamblia intestinalis*.  
(Schewiakoff.)

finely granular, excepting for two rows of larger granules which begin on either side of the nucleus and converge posteriorly. From the anterior part project three or four flagella, which seem to begin at a basal thickening near to, or connected with, the more or less oval, indistinctly vesicular nucleus. From the origin of the flagella an undulating membrane extends backward. The body also seems to possess a certain linear structure connected with the membrane. Contractile vacuoles have not been seen.

**Trichomonas Hominis Davaine.**—This form, found frequently in the human alimentary canal, is very similar to the *Trichomonas vaginalis*, but it is smaller and more pear-shaped. It has been found often in acute diarrheas, but no causal relation between it and the pathological process has been shown.

A similar form has been seen a few times in lung gangrene, aspiration pneumonia, and bronchiectasis.

**Lamblia intestinalis** (Lambl, 1859), a flagellate belonging to this group, parasitic in the small intestines of mice, rats, rabbits, dogs, cats, and sheep, has also been found occasionally in the human intestines. It is beet-shaped, bilaterally symmetrical,  $10\mu$  to  $21\mu$  long and  $5\mu$  to  $12\mu$  wide, possessing flagella  $9\mu$  to  $14\mu$  long. Anteriorly, this species has a characteristic concavity, the rim of which seems to be contractile,

forming a sucking apparatus. The eight flagella of the organism are arranged in pairs: one anteriorly, two laterally, and one posteriorly. The nucleus is situated anteriorly and has a central constriction. The protoplasm of the body is thick and hyaline. Contractile vacuoles have not been seen. Schaudinn (1906) observed encystment, copulation, and complicated nuclear changes in this organism.

Infection follows the ingestion of the cysts with unclean food. The parasites fasten themselves to the free surfaces of the epithelial cells by their sucking apparatus, but seem to exert no harmful influence on their hosts. They have been found most frequently in poor children who play often in dirt containing the cysts. Repeated small doses of calomel will cause their disappearance from the feces.

## CHAPTER XLI.

### AMEBA.

SEVERAL authors have reported the finding of amebæ in man, especially in so-called tropical, ulcerative, or amebic dysentery, but as the first descriptions were incomplete and the laws of nomenclature were not strictly followed there resulted many synonyms for the same species and many species bearing the same name.

At present the four that were considered as distinct species have now become three according to some authors, and only two of those described, from a practical stand-point, need be considered. These are *Entameba histolytica*, the form described by Schaudinn from tropical dysentery and considered by him the cause of that disease, and *Entameba coli*, the kind found in normal human intestines by Schaudinn and thought by him to be harmless.

The discussion in regard to the "parasitic" and the "non-parasitic" forms is still going on. Some investigators say that certain amebæ are strict parasites of the intestinal tract of mammals; that is, that these amebæ cannot be cultivated on artificial culture media, and that they are so different from the "saprophytic" forms, i. e., those that are cultivated (?), that they must constitute a different genus.

Williams, Calkins, and others, on the contrary, believe that not enough study on the "cultural forms" has been done to draw definite conclusions as to their relationship to those seen in stained preparations from amebic dysentery.

**Historical Note.**—Stiles has given a detailed history of the generic name *Ameba* and of the specific one *Ameba proteus*, and, finally, of the naming of the intestinal amebæ. He shows why the name *Entameba* should be given to the genus described by Lambl and Lösch.

This article illustrates very forcibly the absurdity of bringing forth new names for organisms only half studied and of claiming that such organisms belong to new genera.

The first report on intestinal amebæ of man was made by Lambl, in 1860, who announced the presence of ameboid forms in the intestinal mucus of a child who had died from enteritis. Supposedly the same forms were more fully described by Lösch, in 1875, under the name *Ameba coli*. Lösch found his organisms in stools of a patient suffering from chronic dysentery and he succeeded by rectal injections in producing superficial ulceration in the large intestines of dogs. He therefore claimed that this organism is the cause of dysentery. His work was corroborated by many observers. In the meantime amebæ were found in diseases other than dysentery, and Grassi, in 1879, reported them in healthy intestines. The work of Kartulis (1886), however, helped largely to establish the fact that amebæ play an important part in the etiology of dysentery in Egypt. He was the first to find the organism in abscess of the liver in tropical dysentery. In our own country among the most important workers in this field are Councilman and Lafleur (1891). They conclude that amebic dysentery should be regarded etiologically, clinically, and anatomically as a distinct disease. They disapprove, however, of the name *Ameba coli* and propose the name *Ameba dysenteriae* for the pathogenic form; but as they do not show in any way

than by its pathogenesis that the species they describe is a new one, their name, according to the rules of zoological nomenclature, cannot be accepted. Harris's work, too, is important in showing an etiological relationship between amebæ and a certain form of dysentery, but neither did he describe the morphology of his organism minutely enough to identify it with Schaudinn's histolytica which is described below. Casagrandi and Barbagallo, in 1897, were the first to claim that the amebæ so far described in man show differences enough from the fresh-water amebæ to belong to a new genus. They therefore created the genus *Entameba* and gave the specific name *Entameba hominis* to amebæ of the *Ameba coli* type. Schaudinn and Stiles agree with them as to the generic name, but consider that the correct specific name is complicated by the fact that there are different species in this group. Many observers (Kartulis, Councilman and Lafleur, Quincke and Roos, Kruse and Pasquale) have considered that there are different varieties in the human intestines, but they have given no morphological differences distinct enough to classify such varieties. Schaudinn is the first who bases upon a definite morphology his claim (1903) that at least one species among them is pathogenic and one non-pathogenic. The latter, which he found in normal human intestines, he says resembles those already described as *Ameba coli*; therefore he gives it the name *Entameba coli*; while the former, which he found exclusively in ulcerative tropical dysentery, he calls *Entameba histolytica*.

The different views upon the relationship to disease of amebæ found in the human intestines may be summarized as follows:

1. That the amebæ in man have no pathogenic properties, hence are not the cause of amebic dysentery. (Cunningham, Grassi, Celli and Fiocca, Casagrandi and Barbagallo, and others.)

2. That any intestinal ameba may become pathogenic and cause the specific malady known as amebic dysentery. (Musgrave, Clegg, and others.)

3. That amebæ are able to keep up a preëxisting inflammation. This was the original view advanced by Löscher when he described the most commonly cited form, *Ameba coli*, and several authors have followed Löscher in this opinion.

4. That more than one species of amebæ are found in man, at least one pathogenic, and one non-pathogenic. (Kartulis, Councilman and Lafleur, Quincke and Roos, Strong, Schaudinn, Craig, and others.)

The study of bacillary dysentery by Shiga, Kruse, Flexner, and others (see under Bacillary Dysentery) has demonstrated that there are at least two forms of dysentery, one produced by amebæ and the other by bacilli, and from the work on the former it now seems certain that it is produced by a specific form of amebæ.

Amebæ have been reported in teeth cement and in carious teeth as well as in abscesses of the jaw. Flexner, in 1892, described an amebic organism in the latter condition, and considered it identical with the organism described by Löscher, Councilman and Lafleur as *Ameba coli*. In the same year Kartulis described similar organisms found in similar lesions. Gross and Sternberg found them in tartar of teeth. Smith, Barrett, Bass and others have recently found amebæ in practically all cases of Rigg's disease and have claimed an etiological relationship which many other authors have combated. Prowazek (1904) described a mouth ameba as a distinct morphological species under the name of *Entameba buccalis*.

Successful cultures have been made of amebæ obtained from the intestines of man and other animals, as well as from certain fruits and vegetables.

Musgrave and Clegg (1904) studied amebæ in the Philippines by the culture method and came to the conclusion that forms obtained from various sources were probably all a single species.

Williams (1911) obtained pure cultures on sterile "tissue media" of amebæ isolated from the intestines of mammals, including one from a case of amebic dysentery.

**Sites of Amebæ in the Human Body.**—Intestines and neighboring tissues; abdominal cavity; abscess of liver, lung, pleura, brain, and mouth; necrosis of jaw-bone; tartar of teeth; urine.

**Materials and Methods for Study.**—Human cases of dysentery showing amebæ in stools are so seldom on hand in the northern part of this country that they cannot be counted on. The non-pathogenic form in human intestines might be obtained after administration of a saline cathartic, but generally one must depend upon saprophytic forms for work with students, or upon cultures obtained from cases of amebic dysentery. Material rich in saprophytic forms may be obtained from an infusion in water of lettuce, cabbage, potato skins, or other vegetable material. Such an infusion should be made a week or two before it is needed, when it will be found that the pellicle which forms contains many varieties of protozoa and bacteria, among which are generally large numbers of ameboid forms. Often one may get good material from the feces of many of the lower animals, such as the lizard, toad, or guinea-pig.

If one has material containing human intestinal amebæ, kittens or puppies may be fed with the cysts in order to obtain a new supply. The amebæ should be examined in both the fresh and fixed condition. Cultures may also be made as described below.

**Examination of the Fresh Material.**—The study of the living amebæ is extremely important. This may be done by making a hanging drop or hanging mass (p. 72) from fluid containing amebæ. The size, kind of motion, frequency of pulsation of contractile vacuole, and as much of the cell contents as possible should be noted.

The stools should be examined on the warm stage as soon as possible after their passage (not later than two hours), and should be kept at blood heat until examined. A platinum loopful of material should be taken from the slimy masses in the thinner part of the feces, diluted with physiological salt solution, covered with a cover-glass, and examined under moderate magnification.

Harris found that a drop of a watery solution of toluidine blue added to a small particle of the feces stains the entoplasm of the amebæ at once and the ectoplasm a few minutes later. The amebæ seem to be quickly killed and often when natural forms are beautifully preserved the preparations, after being washed in water and mounted in Farrant's medium, may be preserved for months, but after a time the stain completely fades.

**Permanent Preparations.**—Thin films are made on glass slides or cover-glasses, and immediately, before they are allowed to dry, they are placed in the fixing solution. Cover-glasses may float film down, on the surface of the fixative. Among the best fixatives are: Hot sublimate alcohol (50° C.), Zenker's fluid, hot Hermann's fluid, or methyl alcohol (p. 85).

**Stains.**—Several of the many good staining methods are given in the chapter on Stains.

1. Thin Delafield's hematoxylin, from one-half hour to several hours, then washed in water. (If overstained, the preparation may be differentiated in acid alcohol, controlling under the microscope, then washed in water.) The film or section is then passed successively through 70 to 95 per cent. and 100 per cent. alcohol, absolute alcohol + xylol, xylol, cedar oil, or Canada balsam.

2. Heidenhain's iron hematoxylin (see p. 84). The smear is changed from distilled water into the iron-alum mordant for four to twelve hours, or overnight; well washed in distilled water; in stain from two to twenty-four hours, excess washed out in the iron mordant, controlled under the microscope (as decolorization occurs very quickly) until the nucleus is sharply differentiated; the chromatin of the nucleus must be a deep blue black, and the cytoplasm a light gray; then a thorough washing in tap water and passage through the alcohols and xylol, and in Canada balsam, or cedar oil for mounting.

3. After fixation in methyl alcohol one may use Giemsa's staining method (see p. 82), or a modification of the method suggested by Van Gieson for staining the Negri bodies in smears (see p. 83).

Masses containing amebæ, as mucous flakes or portions of the intestinal or liver abscess wall in amebic dysentery, or pieces of decaying vegetables may be fixed *in toto* in hot sublimate alcohol for one-half hour, washed in iodine alcohol for twenty-four hours, passed through the different strength alcohols and imbedded in paraffin (see p. 85) for section cutting if desired.

**Cultures of Amebæ.**—Pure mixed cultures may be made in the following way: From the material containing amebæ a small loopful is removed with a platinum wire and isolated spots are touched over the surface of ameba agar (see p. 111) poured into sterile Petri dishes. If necessary, feces may be thinned with physiological salt solution before planting. In one to several days at 25° C. the amebæ with the accompanying bacteria may overgrow the entire plate. We have found that amebæ will grow as well upon nutrient agar—better with certain bacteria—as on the special medium just mentioned. Impression films may be made of these cultures, or small pieces of agar and culture may be imbedded entire. From such a culture the “pure mixed” cultures of Frosch may be made as follows. The amebæ which have crept out to the periphery of the growth are taken out with their accompanying bacteria and transplanted. Usually one or two organisms favorable for the growth of the amebæ accompany them and in this way one may finally get the amebæ growing with one definite bacterium. We have isolated from a culture a single ameba unaccompanied by bacteria by the following simple method: Under the low-power lens with a fine platinum loop an isolated ameba is drawn to the edge of the agar plate. When it is well separated a disk of agar containing it is cut out following the margin of the objective and is transferred to a fresh agar plate. A very small quantity of a desired bacterium is now added to the disk near the ameba, and a “pure mixed” culture results.

**Pure Cultures.**—Certain varieties of amebæ from the intestines of mammals grow without other organisms, *i. e.*, grow in pure cultures, when inoculated on a piece of fresh sterile brain, kidney or liver placed upon Musgrave and Clegg’s ameba agar or on nutrient agar. They grow abundantly on such media at temperatures varying from 22° C. to 38° C. (Williams).

**Morphology.**—The morphological characteristics of the ameboid stage, as described by various observers, seem not to have been minutely enough studied to be depended upon in differentiating the species. Moreover, descriptions have differed markedly. While Schaudinn and others, especially Craig of the more recent writers, say that it is easy to differentiate between the ameboid forms of *histolytica* and *coli*, Musgrave and Clegg, Strong, Williams and others say the points of difference are not marked.

The observations of Schaudinn and others may be summarized as follows: (1) *Ent. coli* is, on the whole, smaller than *Ent. histolytica*; (2) its ectoplasm is so small in amount and so slightly differentiated that it is only seen when the organism puts forth pseudopods, while the cortical zone of the *Ent. histolytica* is wider and is distinctly differentiated from the entoplasm; (3) the pseudopods of the former are small, rounded, delicate, and not highly refractive, those of the latter are larger, finger-shaped, firmer, and more highly refractive, thus indicating the power of the organism to penetrate its host’s tissues; (4) the nucleus of *Ent. coli* is very distinct in life as well as in stained spreads, due to a definite membrane, a more distinct karyosome, and much chromatin which is distributed throughout the nucleus with more of a collection



about the periphery; the nucleus of *Ent. histolytica* on the other hand, is seen with difficulty during life, and stains faintly, owing to its delicate membrane, its small amount of chromatin, and small karyosome; the chromatin is collected about the karyosome and the periphery of the nucleus; the nucleus, moreover, is much more variable in shape, in the active organism than is that of the *Ent. coli*; (5) the entoplasm of *Ent. coli* is less granular and vacuolated and contains fewer red blood cells than that of *Ent. histolytica* which sometimes shows immense numbers of these blood cells (Plate IV, Fig. II, 1-5).

The above points of difference cited for organisms in the ameboid stage may hold for forms living in the human intestines; but we have found that organisms from widely different sources (*e. g.*, intestines of guinea-pigs and of dogs from New York and of humans from the Philippines) when grown with a favorable bacterium in the thermostat at body temperature may show appearances similar to each other and similar also to those described by Schaudinn for *Ent. histolytica*. More corroborative work, therefore, seems to be needed before we accept the above observations as being the whole truth.

**Reproduction.**—In the vegetative stage probably all these forms divide by a primitive mitosis, though Schaudinn, Craig, and others saw only amitosis. All of our culture forms divide by mitosis, and many observers have recently reported similar division in related forms. Schaudinn and others state that *Ent. coli* in the vegetative stage may divide by breaking up (schizogony) into, at the most, eight daughter cells. In the latter instance, according to these authors, the nucleus undergoes a somewhat complicated process of division. But recently Walker, Hartmann, and Williams have presented evidence to show that this is probably not true. The nuclei continue to divide by binary mitosis.

The vegetative stage of each intestinal organism takes place in the upper part of the intestines; as the feces become thicker most of the vegetative forms die off, while some pass on to permanent cyst formation. As with many coccidia, parasitic amebæ may pass through a long period of vegetative life before entering upon another phase wherein forms are produced capable of infecting a new host. The length of this period depends upon a number of circumstances. Under conditions favorable for the growth of the amebæ, as in cases of diarrhea, the vegetative phase is considerably lengthened, while in healthy intestines, as the amebæ pass down with the thickening feces, the infecting cysts are more or less quickly formed.

The statement made by Schaudinn that *Ent. histolytica* during the vegetative stage may multiply by budding as well as by binary fission, is now considered incorrect. Unequal division, however, probably frequently occurs.

It is now thought by Walker, Hartmann, Craig, and others that *Ent. tetragena*, described by Viereck, Hartmann, and Craig as a distinct species, is merely a phase of *Ent. histolytica*, though Hartmann calls the species *tetragena* and drops the name *histolytica*.

The observations of Schaudinn on the sexual phenomena of *Entamebæ*, though earlier corroborated by Hartmann, Craig, and others, are now considered incorrect by these same observers. The subject awaits further investigation.

**Viability.**—The pathogenic amebæ are apt to lose their motility very quickly above or below body heat, while the saprophytic forms remain motile at higher or lower degrees. Though the former lose their motility, they are not all killed by cold. They may still be infective after freezing. Musgrave kept an encysted culture from a dysenteric stool at  $-12^{\circ}$  C. for 45 days and found it still viable at the end of that time.

A temperature of  $60^{\circ}$  C. for one hour usually kills encysted cultures of amebæ, according to Strong, but considerable variation has been noted in the degree of temperature necessary to destroy different strains.<sup>1</sup>

Enemata of quinine sulphate and saturated solution of boric acid do not affect amebæ in the intestinal canal, though a 1 to 300 solution of quinine sulphate added to the stools invariably kills them in ten minutes.

They are also destroyed in stools by weak solution of hydrogen dioxide, potassium permanganate, toluidine blue, and dilute acids.

Little found that 1 to 10000 hydrochloric acid and 1 to 100 silver nitrate check motility, but do not destroy parasites except after prolonged contact. Musgrave and Clegg found that in cultures treated with 1 to 2500 solution of quinine hydrochlorate the parasite quickly encysts, and in from five to eight minutes may break up and disappear; ten minutes later cultures made produced no growth of amebæ, while the bacteria<sup>2</sup> grew well. Emetin has a marked effect *in vitro* and *in vivo* (see below).

**Pathogenicity.—Lower Animals.**—Just how pathogenic *Ent. coli* is for lower animals cannot be determined, as we have before stated, until a more minute study of the intestinal amebæ is made.

In regard to amebæ from tropical dysentery (presumably *Ent. histolytica*), they have been shown to be pathogenic for young cats, dogs, and monkeys. The infection may take place in two ways: (1) By feeding material containing the cysts; (2) by rectal inoculations of the vegetative forms. The best work done on dogs is by Harris, in 1901, who found that puppies were particularly susceptible after rectal injections of fresh material from human dysentery cases. Morphine was administered before the injection in order to retard peristalsis. The disease developed in two or three days and lasted from four to sixteen days.

The chief symptoms were a bloody diarrhea and progressive emaciation. The lesions observed in the intestines on postmortem examinations were a swollen and congested mucosa, over which were scattered numerous small ulcers. In two cases there were liver abscesses.

Microscopically the mucosa first showed slight exudative and productive inflammation, followed by necrosis and desquamation of the epithelial cells and their basement membrane. At the same time the interglandular tissues beneath became swollen and small hemorrhages occurred. Great numbers of

<sup>1</sup> An air-dried agar plate culture of "*Ameba coli*" given us by Dr. Calkins who obtained it from the Philippines was viable after three years at room temperature kept in the dark.

macrophages collected. Ulceration proceeded from above downward. Many amebæ were first seen in and between the epithelial cells, then in the connective tissue at the base or sides of the ulcers. Necrotic and suppurative processes producing varying degrees of suppurative inflammation may complicate the lesions.

The abscesses which form in the liver contain degenerated liver cells, polynuclear leukocytes, red blood cells, and groups of small amebæ.

As controls Harris tried rectal injections of various bacteria, including the Shiga bacillus. All gave negative results, however, and he considered that the amebæ showed their specific action very plainly. Though he did not describe the morphology of the organism from his cases with enough minuteness to identify it with Schaudinn's *histolytica*, he gave enough points to make the inference strong that it is the same species. Whether *Entameba coli* would produce similar dysentery in young dogs is yet to be proved. As stated above, Schaudinn found that he could produce the typical disease by feeding young cats with cysts of *Ent. histolytica*, but could not get the same results by feeding the vegetative forms.

Musgrave and Clegg injected "pure mixed cultures" of material from cases of clinical amebic dysentery as well as similar cultures of amebæ from various sources into monkeys and produced dysentery. Musgrave fed monkeys with encysted amebæ in bacterial cultures and obtained, in a small percentage of the cases, dysenteric stools and ulcerations in which amebæ were found without their accompanying bacteria. Kartulis, Kruse and Pasquale, and Strong injected into the rectum the contents of liver abscesses containing apparently only the amebæ and produced typical dysentery, with lesions similar to those seen in man.

Strong states that the lower monkeys and the orang-outang in the Philippines contract the disease naturally.

**In Man.**—According to Craig, about 50 per cent. of human beings harbor harmless amebæ in their intestines. Schaudinn states that he found this form of ameba in one-half the cases examined in East Prussia, one-fifth of those in Berlin, and 256 times in 385 examinations in Austria.

Recently Walker and Sellards have carried on an extensive study of the pathogenicity of the different forms in human beings—60 Philippine prisoners—which they divided into three groups:

1. Twenty men fed with cultures of amebæ isolated from stools or Manila water. The same ameba was recovered in cultures from several of the cases. No lasting parasitism occurred, neither did any case of dysentery result.

2. Twenty men were fed stools containing *Ent. coli*. No cultures were recovered; no cases of dysentery occurred, but 17 cases were parasitized.

3. Twenty men were fed stools containing *Ent. histolytica*. No cultures were recovered; 4 cases of dysentery occurred, and 17 cases were parasitized.

They conclude from these studies that they have furnished the proof of the pathogenicity of one form (*Ent. histolytica*), the non-pathogenic but parasitic nature of the other form (*Ent. coli*), and the saprophytic nature of cultural forms. These experiments are open to a number of criticisms, chief of which are: Probable immunity of the men, the use of only Musgrave and Clegg's culture medium for their cultures, and their cultivation only at room temperature.

The disease produced by pathogenic amebæ in man is known as *amebic dysentery* (amebic colitis, amebic enteritis, amebiasis entamebiasis).

**Incidence.**—The disease occurs endemically in tropical countries. It is particularly prevalent in Egypt, India, and the Philippine Islands. It occurs frequently in parts of South America and southern United States. In northern United States few cases are reported, though Patterson, who in 1909 described three cases (without a description of the amebæ present), and who calls attention to fifteen other cases reported as endemic in New York City since 1893, states that this disease is probably more widespread than is generally thought, and that if it were searched for more carefully more cases would be recognized. Patterson adds to his report a bibliography of cases reported as originating in North America. Sporadic cases are found in Russia, Germany, Austria, Italy, and Greece. An occasional small epidemic may occur in the milder climates. Where it is endemic the largest number of cases occur after the heavy rains have begun in early summer. Males are more frequently attacked, because more exposed to infection. It may occur at all ages, but young adults seem most susceptible. The foreign white race seems to be more susceptible than natives. Unhygienic surroundings are generally a predisposing factor, but in the Philippines individuals of all classes are likely to be attacked who do not take continuous and extraordinary precautions in regard to their drinking water.

**Symptoms.**—The symptoms may be mild or severe. The disease usually runs an irregular course marked by periods of intermission and exacerbation. It may begin acutely with slight fever, griping, tenesmus, and frequent stools. Occasionally, however, the outset is gradual, lasting from a few days to several weeks. The disease is generally chronic, extending over a period of a few weeks or of many years. In the mild form which is usual in children, the general condition may be remarkably good, the only symptoms worth mentioning being the increased number of stools—two to six in twenty-four hours—which contain few to many amebæ. In the severe forms there is a loss of appetite, great emaciation, some fever, acceleration of the pulse, sweating, abdominal pains, and a decided increase of the number of stools—twenty to sixty daily. The stools are more fluid and slimy and may be bloody. They contain amebæ in varying numbers. In very severe forms the stools are watery, filled with blood, mucus, and sometimes sloughs. They vary in numbers from twenty to fifty in twenty-four hours and may contain many amebæ.

The milder forms may change suddenly to the severest, and the severest may suddenly become better and completely recover.

**Tissue Changes.**—The lesions are chiefly in the large intestines. The walls are thickened in chronic cases, especially the submucosa. There are raised hemispheric areas of hemorrhagic catarrh and of ulceration. The whole of the large intestines may be affected or only more or less circumscribed areas. The amebæ pass between the epithelial cells, generally through small erosions, and they finally reach the submucosa by the lymph channels. Here reproduction takes place and the irritation to the tissue causes edema and infiltration of small spheroidal cells. This produces small elevations into the lumen of the intestines. The epithelium over these raised areas is finally eroded and then bacteria and intestinal contents help form the succeeding ulcers. The erosions or ulcerations have congested undermined margins, and yellowish-red bases. They vary in size from 2 mm. to about 2 cm. They are round, oval, or irregu-

lar in outline. The ulceration usually extends only to the submucosa, but may expose the peritoneum, and large sloughs may be cast off into the lumen of the intestines. Generally the slow inflammatory process in the submucosa leads to great thickening of the intestinal wall.

The processes may be modified in various ways by the action of other micro-organisms, especially the bacteria in the feces. Healing takes place by the formation of connective tissue in the floors and by a gradual covering over with epithelium. In extensive lesions scars may form.

Peritonitis may occur with the production of an opaque gelatinous fibrinous fluid in which the amebæ may be found.

Abscesses may form in the liver (about 20 per cent. of all cases), less often in the lungs, and only occasionally in the brain and spleen. Amebæ may reach the liver through lymph channels, portal vein, and peritoneal cavity. The other organs are only slightly changed.

**Source of Amebæ.**—Nothing can yet be said about the exact source of Schaudinn's pathogenic variety, as so few have identified the organism. Strong states that in Manila the greatest source of infection from amebæ is the water supply, that amebæ were cultivated from the water in large numbers in 1902, but no attempt was made to demonstrate their pathogenicity. In 1904, however, Musgrave produced dysentery in a monkey with a culture of a water ameba, though in a few experiments he was unable to infect cats from the amebæ obtained from this monkey. Practically, it is proved that people in Manila avoid being infected with amebæ if they do not drink local water, unless sterilized. Fresh vegetables as well as certain fruits may be sources of infection. Of course if pathogenic amebæ are strict parasites, the source would be only those substances contaminated with the host's excretions.

As dilute acids quickly kill the motile amebæ, it is probable that many of those ingested in this form are destroyed in the stomach.

**Immunity** to the disease may exist. It is supposed that the amebæ as they die produce toxic substances which call forth antibodies, but this has not yet been determined. The necrosis produced in the liver abscesses when bacteria are absent is an indication of the production of necrogenic substances.

**Prognosis.**—The percentage of deaths in the severe cases is quite large, especially if accompanied by abscess of the liver. Probably 25 per cent. of all cases are fatal. When treatment is begun early the prognosis is better.

**Treatment.**—Emetin has been found to be practically a specific curative treatment when amebæ are in the vegetative stage, but it does not affect cysts. In the form of ipecacuanha it is highly recommended by Manson, Dock, and others, especially since the introduction of salol-coated pills which allow the remedy to reach the intestines before it is absorbed, so that large doses may be given, without inducing marked nausea and vomiting. Besides rest and diet, high enema of bisulphate of quinine have been recommended. Harris has gotten good results from hydrogen dioxide enemata diluted from 4 to 8 times with water. About a quart is injected twice daily for a week, then the amount is gradually decreased.

**Points in Diagnosis of Amebæ Found in Man.**—Examination of stools should be made as quickly as possible after they have been passed and they should be free from urine. The amebæ should be seen motile because, after encystment or death, it is often difficult to distinguish them from other intestinal contents. Bloody mucus or small pieces of necrotic tissue should be examined first, as they often contain large numbers of amebæ.

If the movements are solid a dose of salts should be given and the fluid part of the resulting stools examined.

Craig differentiates living pathogenic forms from non-pathogenic varieties by the former's (1) larger size, (2) greenish color, (3) distinct hyaline, refractive ectoplasm, (4) faint nucleus, (5) many vacuoles and red blood cells, (6) marked motility.

An absolute diagnosis of liver abscesses can often only be made by an exploratory puncture and the finding of the amebæ. If this is done the surgeon should be at hand to operate if necessary.

*Ent. buccalis* is usually found in the thick group of leukocytes and microorganisms collected between the teeth. The amebæ are distinguished from the leukocytes and cell detritus by (1) their large size, (2) their light, highly refractive greenish appearance, (3) their glistening red color in contrast to the yellowish red of the leukocytes when hanging drops are stained with enough of a concentrated solution of neutral red to make them appear pink.

**Differential Diagnosis between Amebic and Bacillary Dysentery.**—In amebic dysentery (1) the disease is generally chronic; (2) dysentery bacilli are usually not found in feces; (3) no severe toxic symptoms present; (4) abscess of liver frequent sequela; (5) lesion is in cecum and descending colon, not in small intestines.

In bacillary dysentery, the finding of the bacilli, and a positive agglutination test, together with the clinical symptoms of intoxication, make a positive diagnosis.

## AMEBÆ IN OTHER DISEASES.

Baelz found a very large ameba in the bloody urine and in the vagina of a twenty-three-year-old Japanese who was suffering from tuberculosis of the lung. Jurgens, Kartulis, and Posner also reported finding similar amebæ in cases of cystitis and bloody urine.

In the last few years many studies have been made on the amebæ present in pyorrhea alveolaris. The claim of Smith, Barrett, Bass and others that a specific ameba is the cause of this condition has not been fully substantiated. In examining many school children we found that similar amebæ in large numbers are found in the majority of cases, but no definite relation could be traced between them and the beginning of pyorrhea.

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## CHAPTER XLII.

### SPOROZOA. CILIATA.

THE forms in the group sporozoa, which are parasitic in man, or which are of some medical interest, are, *Coccidia*, *Nosema*, *Sarcocystis*, *Babesia*, and *Plasmodium malarie* and its allies. *Babesia* and the malarial organisms will be considered in separate chapters.

Only one genus among the ciliates is pathogenic for man.

The general characteristics have already been described in Part I.

#### EIMERIA STIEDÆ (COCCIDIUM CUNICULI).

The *Coccidium cuniculi* is a sporozoan parasite of the rabbit first described by Lindemann in 1865. Young rabbits are especially susceptible, and extensive epidemics may occur in breeding houses.

**Material and Methods for Study.**—Rabbits infected with *Coccidium cuniculi* are often found, and the whole course of the infection may be followed with more or less ease.

A certain amount of development may be watched in hanging drops of salt solution emulsions. Sections and smears are prepared as described in Chapter III.

The cysts are stained with difficulty. It is recommended that a thin solution of Delafield's or Grenacher's hematoxylin be used for twenty-four hours followed by eosin. Heidenhain's iron-hematoxylin stain (p. 84) followed by Bordeaux red is especially good for sections.

The *symptoms* of the disease are fever, diarrhea, yellowish mucous discharge from the nose and mouth, and progressive wasting. The liver is much enlarged and shows throughout its substance variously sized grayish-white tubercles, generally surrounded by a capsule, and containing a slimy mass of degenerated host cells, in which the parasites are imbedded. The parasites are also found in the feces and in the epithelial cells of the intestines, gall ducts, and liver. The acute stage of the disease lasts about three weeks. The contents of the coccidial tumors in animals that have withstood the infection may later be emptied, leaving only a mass of cicatricial tissue. In such animals the oöcysts may remain for a long time in the gall-bladder and intestines, and by passing out gradually with the feces may provide a source of infection for other animals. The infection is carried by food soiled with cyst-containing feces. The cysts pass with the food into the stomach, where the cyst wall and the spore sac are destroyed and the sporozoites are set free. The motile sporozoites pass through the ductus choledochus into the liver, some probably passing into the intestines and infecting the cells directly, a later infection of the intestines occurring from forms developed in the liver. The organism develops within the epithelial cells of the liver and gall ducts until the cells are finally broken down and tissue cysts are formed, within which, after more or less complicated changes, cysts of the parasite are again formed. The life cycle of *Eimeria Schubergeri*, a similar coccidium from the intestines of a myriapod, is shown on Plate IV, Fig. III, A, 1-16.

A few cases of human infection of the liver with the *Coccidium cuniculi* have been reported. The *Coccidium hominis* Rivolta, found a



few times in the human intestines, as well as similar coccidia, found in the intestines of lower animals, may belong to the same species.

*Coccidium bigeminum* (Stiles) is found in the feces of dogs, cats, polecats, and possibly human beings. The organism is characterized by the division of the oöcyst into two united cysts, containing four spores. The size is  $8\mu$  to  $15\mu$ . The life cycle is not well known.

*Rhinosporidium kinealyi* is the name given by Minchin and Fantham (1905) to a probable sporozoan found in the nasal mucous membrane of certain cases from India that were troubled with hemorrhagic nasal polyps. Nais reported four similar cases and Beattie another in 1906.

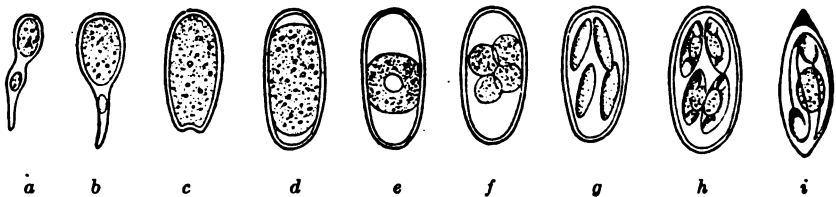


FIG. 180.—Showing spore formation in *Coccidium cuniculi* from the liver of the rabbit: a and b, young stage in the epithelial cells of the gall ducts (the small oval is the cell nucleus); c, d, and e, the fertilized oöcyst; in d the protoplasm is beginning to shrink away from the cyst wall, and in e it has contracted into a spherical form; f, segmentation into four sporoblasts; g, elongation of the sporoblasts to form spores; h, four complete spores in the oöcyst; i, single spore more highly magnified, showing the two sporozoites and a small quantity of residual protoplasm. The life cycle has been fully worked out by Simon. (After Balbiani, from Doflein.)

### MYXOSPORIDIA.

The *Myxosporidia* belong to one of the most populous and abundant groups of the sporozoa. They show great structural variation as well as divergence in

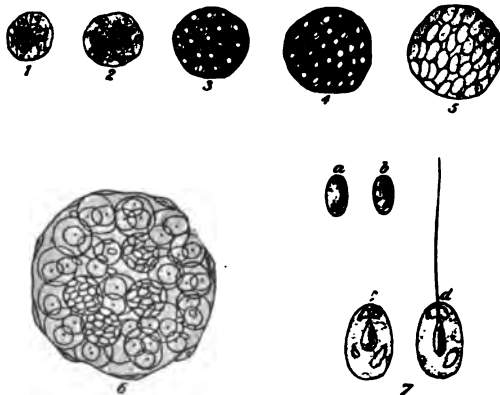


FIG. 181.—*Nosema bombycis*: 1 to 5, spore formation; 6, infected follicle of testicle; 7, spores; a, b, fresh; c, d, treated with nitric acid. The acid causes them to swell up and increase in size by at least a half, at the same time making the polar capsule distinct. In d the filament is extruded. (After Balbiani.)

mode of life, nevertheless the members have, as a group, the following well-marked characteristics: The trophozoite is ameboid; spore formation begins at

an early period and proceeds continuously during the growth of the trophozoite; the spores are produced endogenously—i. e., within the protoplasm of the trophozoite, and each spore always possesses one or more very distinctive structures, "the polar capsules" (Fig. 181, c, d).

The myxosporidia are habitants of fishes, reptiles, arthropods, and some other classes of animals. They infest especially arthropods, causing often most virulent epidemics. The most interesting member of this group is *Nosema bombycis*, the cause of silkworm disease (Pébrine). The organism forms many small spores each with one polar capsule. The spores, which are carried by the food into the intestinal canal of the caterpillar, pass through the walls of the intestines, and infect all organs. Spores found in the ovary may be carried over to the newly hatched silkworms, thus causing a further dissemination of the disease.

As far as is known man is not infected by this group.

The other member of this group, of interest here, is *Nosema lophii* Doflein. Its interest lies in the fact that it has been found to infect only the ganglion cells of the sea-devil, thus apparently resembling in its parasitic nature the organism causing hydrophobia.

### SARCOSPORIDIA.

This order is very little known but, considering the fact that through eating uncooked infected meat it may be found in man, though rarely, its chief characteristics should be noted here.



FIG. 182.—*Sarcocystis tenella* from the striped muscle of a swine. A full-grown cyst, showing readately striped membrane, which is broken on the right side. (Bertram.)



FIG. 183.—*Sarcocystis miescheri*, a, small cells from a cell group; b, loosening of the protoplasm from the cell wall; c, d, sickle-shaped bodies (sporozoites) formed from the small cells. (From Wasielewski.) (Mans.)

The *Sarcosporidia* are parasites of the striped muscles or connective tissue of some of the warm-blooded vertebrates (various birds and mammals). They are found in the adult state in elongated sacs known as "Rainey's" or "Miescher's tubes" (Fig. 182).

The trophozoite is a motionless elongated body, limited by a cuticle growing into a complicated structure. Spore-formation begins at an early stage

and proceeds during the growth of the trophozoite (*Neosporidia*) which may become very large. The spores, which are many, are minute sickle-shaped or spindle-shaped mononucleate bodies with a delicate envelope and at one pole an oval striated body which represents the polar capsule found in the myxosporidia (Fig. 183).

In some cases the cyst wall calcifies and the contents of the cyst degenerate, with apparently no harm to the host; in other cases the cysts burst and their contents spread into the surrounding tissue, producing abscesses and tumors as with many myxosporidia, and sometimes causing the death of the host.

The symptoms of sarcosporidiosis in the pig are paralysis of the hind extremities, a skin eruption, and general systemic symptoms, such as increased temperature and pulse.

In sheep especially the disease often causes fatal epidemics. In the mouse *Sarcocystis muris* is a deadly parasite. Theobald Smith showed that gray and white mice may become infected with *Sar. muris* by eating infected mouse flesh containing motile sporozoites.

Laveran and Mesnil claim to have extracted a toxin (*Sarcocystin*) by means of glycerin or salt solution, which they have found extremely toxic for experimental animals (0.0001 gm. kills 1 kgm. of rabbit). The dried and powdered extracts are also virulent. These extracts will remain virulent for a long time in the ice-box, but will not withstand heating above 60° for any time.

Darling (1909) describes a case of human sarcosporidiosis occurring in Panama, from which he studied the organism and came to the conclusion that it was probably a different species from the one already described as occurring in man. He gives a good historical review. Later he decides that morphologically his human sarcosporidia are identical with *Sarcocystis muris*.

### CILIATA.

***Balantidium coli*** (Malmsten, 1857). The body of this infusorium is egg-shaped, with a funnel-shaped mouth opening. The surface of the body is covered with a pellicle, under which is a distinct ectoplasmatic sheath containing rows of basal granules from which short, fine cilia arise.

The cloudy entoplasm contains fat and starch granules and may contain many red blood cells and other food particles from the host. Two contractile vacuoles have been seen. Posteriorly there is a small prominence marking the place where excreta are expelled. The chromatic macronucleus is bean-shaped, and the vesicular micronucleus is nearly spherical (Plate IV, Fig. iv, A-D).

Division is transverse, the macronucleus dividing by simple constriction and the micronucleus by mitosis. Conjugation has been observed. Spherical cysts surrounded by a thick membrane are formed.

*Balantidium coli* has been found in the large intestines of human beings and of swine—probably two distinct varieties. The variety occurring in human beings has been found in about 60 cases, principally in Sweden, but also in Russia, Scandinavia, Finland, China, Italy, Germany, the Philippines, and the United States. Most of these cases were suffering from severe chronic intestinal catarrh, often accompanied by bloody diarrhea. A number of observers (Strong, Brooks, and others) think the balantidium the primary cause of the catarrh, while others believe it to be a harmless inhabitant of the intestines, or at least only a secondary excitant (Opie, Malmsten, Doflein, and others).

Schaudinn has described two additional species of balantidium found in the human intestines, which he has called, respectively, *Balantidium minutum* and *Nyctotherus faba*, probably both non-pathogenic.

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## CHAPTER XLIII.

### THE MALARIAL ORGANISMS. BABESIA.

**Introduction.**—The malarial organisms are a group of protozoan parasites found to be the cause of a definite group of specific infectious fevers in man, called by the somewhat misleading term malaria, a term which signifies "bad air."

They are classed as sporozoa, order hemosporidia, and are considered by the majority of observers as forming one genus, plasmodium or hemebeba.

So far as is known the only natural means by which the malarial organisms are transmitted to man is mosquitoes, genus *Anopheles*. A part of the life cycle of the organisms is carried on in these mosquitoes. Experimentally the disease has been produced by direct inoculation of infected human blood into humans. Accidentally it has been conveyed in transfused blood. The parasites develop in man within the red blood corpuscles which they finally destroy, thus producing the anemia and pigment granules peculiar to malarial fevers.

**Historical Note.**—The fevers caused by these organisms were recognized and studied as early as 400 B. C., but it was not until 1880 that the true nature of the dancing pigment which had been observed long before was determined. At that time Laveran announced that he had discovered a parasite in the blood which he claimed was the cause of the disease and he published a good description of several of the stages in the life of the organism. The public remained at first almost entirely unconvinced of the parasitic nature of these bodies. Many still believed that the bacillus described shortly before by Klebs was the cause of the fevers. Among others, Marchiafava and Celli in Italy believed that Laveran's organisms represented areas of degeneration within the red blood cells, though Laveran himself demonstrated the organisms to them. When they began, however, to study the fresh tissue themselves they changed their opinion and later they published a number of valuable contributions on this subject. They gave the organism described by them the inappropriate name, *Plasmodium malariae*. Laveran's researches were later confirmed by many other observers.

In 1885 Golgi showed that quartan fever depends upon a specific form of the parasite, and that the malarial paroxysm always coincides with the sporulation or segmentation of a group of parasites. Thus in a single infection with the quartan variety a paroxysm occurs every fourth day, with a triple infection on successive days, segmentation with its accompanying paroxysms occurs daily. Golgi and others soon showed that tertian fever and estivo-autumnal fevers were each due to a distinct variety of the hemebeba. These varieties are at present regarded by some as distinct genera, by others as species, belonging to a single genus. Councilman first called attention to the diagnostic value of the different forms which appear in the blood.

Though it had been thought for nearly two thousand years that malaria is transmitted by insects, the question was not definitely settled until Ross, in 1896, clearly demonstrated that the hematozoa of birds were transmitted by a certain species of mosquito. These investigations of Ross were soon confirmed

by Grassi, Bignami, and others. Manson proved (on his son) that mosquitoes carried the infection to man. MacCallum's observations on the sexual forms of halteridium were a great advance, and Bignami, Grassi, and others soon proved that all varieties of malarial fevers are transmitted from man to man by mosquitoes of the genus *Anopheles*. Grassi worked out the complete life cycle of the pernicious type (estivo-autumnal), while Schaudinn (1901) did the same for the tertian form. In the later classifications the genus name *Plasmodium* has been changed to *Hæmameba*.

**Materials and Methods for Study.**—If a case of malaria is at hand the organism may be examined alive under the microscope by allowing a cover-glass to drop gently upon a drop of fresh blood placed upon a clean glass slide. For finer differential points, however, smears should be made (see p. 73), which may be stained by any one of several methods, such as that of Jenner, Giemsa, or Wright (see pp. 82 and 84 for details of staining). Bass and Johns state that by centrifuging at high speed defibrinated or extracted blood containing malarial organisms, the protozoa rise to the top of the cell column and by this means a mass of crescents or other forms can be obtained.

**The Parasite.**—Three distinct species of malarial organisms in man have been described: *Hæmameba vivax* (causing tertian fever), *Hæmameba malariae* (causing quartan fever), and *Hæmameba falciparum* (*Laverania malariae*, *Hæmomenas præcox*, causing estivo-autumnal fever). The last species has been divided by certain authors into two varieties, a quotidian and a tertian, but it is the present opinion that only one species is definitely known. See table of the chief differences between these forms (also see Plate VII).

#### EXPLANATION OF PLATE VII.

Partly schematic. Drawn and rearranged by Williams, partly from Muir and Ritchie, partly from Kolle and Hetsch and partly original. Giemsa's stain.

The asexual forms show cycle of the organism in the red blood cells of the human host. They show schematically the time of fever and the day of segmentation.

##### *Tertian type.*

FIG. 1.—Segmented organism.

FIG. 2.—Young ring form in cell and a young form on surface.

FIG. 3.—Growing schizont; irregular form due to great motility; beginning pigment formation; red blood cell becoming paler.

FIG. 4.—Larger schizont with dividing nucleus. Red blood cells pale and stippled.

FIG. 5.—Nucleus divided into four clumps.

FIG. 6.—Further division of chromatin and formation of irregular rosette. Pigment finely granular in centre.

FIG. 7.—Segmentation. Note eighteen merozoites (usually sixteen).

*Quartan type.* Shows following differences from tertian. Slightly larger, fewer segments (usually eight), and more regular. Pigment coarser. Red blood cells unaltered. Segmentation every seventy-two hours.

*Estivo-autumnal type* Shows following differential points: Merozoites smaller and more numerous (thirty-two?); organism less motile with less pigment. Red blood cells smaller and greenish color (in fresh cells).

**Sexual Forms.** Show cycle of development in mosquito.

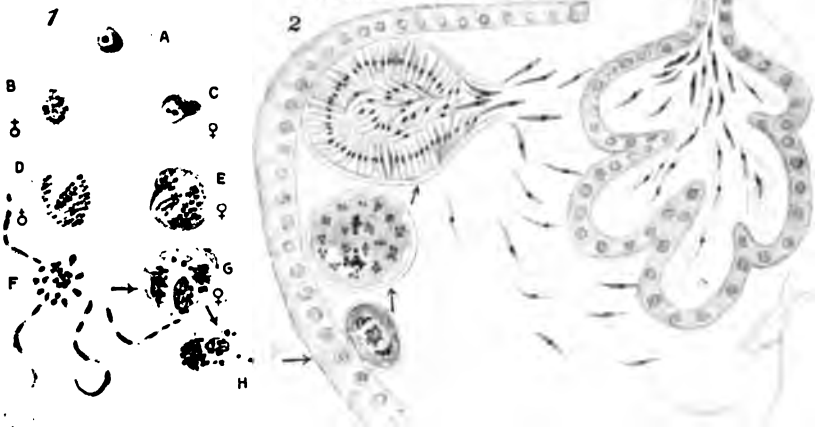
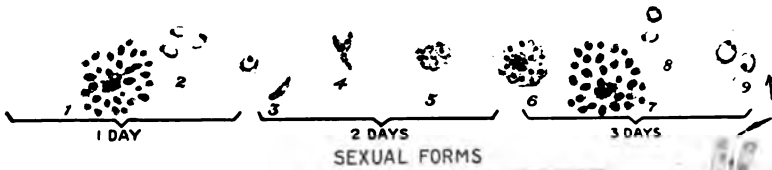
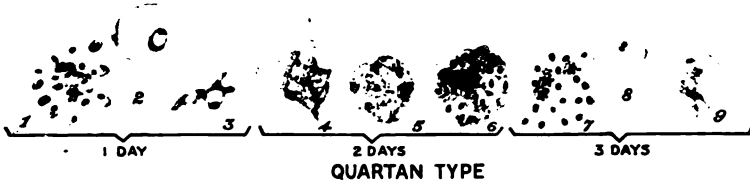
FIG. 1 (A to E).—Male (♂) and female (♀) forms of tertian type formed in human blood; F, flagellation of male type in stomach of mosquito; G, H, changes in female type and fertilization in stomach of mosquito.

FIG. 2.—Development of sporocyst within mosquito. Liberation of sporozoites, which find their way to the salivary gland.

FIG. 3.—Sexual forms of estivo-autumnal type found in human blood, showing development of sickle-shaped bodies.

# PLATE VII

## ASEXUAL FORMS OF MALARIAL PARASITES (SCHIZOGONY) TERTIAN TYPE





Each of these species undergoes the two phases of development already alluded to, one within the red blood cells of human beings (the asexual phase); the other within the digestive tract of the mosquito (the sexual phase). The form changes which each parasite undergoes in humans and which the benign tertian undergoes (which may be considered a type for all) in both hosts are shown on Plate VII. Briefly, they may be described as follows:

**The Asexual Cycle (Schizogony) Occurring in the Blood of Man.—**

The young form is often difficult to find in fresh blood. A pale area is seen on an otherwise unaltered red corpuscle, situated usually eccentrically, about one-tenth the size of the red corpuscle or about one-fourth its diameter, when at rest presenting a rounded appearance, but usually actively ameboid, throwing out distinct pseudopodia never remaining long in the same focal plane, frequently dipping, so to speak, into the substance of the corpuscle. It is often called the hyaline form because it is free from pigment, but it is not hyaline in the proper sense of the term. It is also called the ring form, because of its resemblance to a ring in stained preparations; but it is never a true ring. The ring appearance is produced by the formation of a large food vacuole. The organism continues to grow either on or in the red blood cell.

Rowley claims that the organisms never enter the red blood cells. They only feed from the surface. Her beautiful demonstrations show that possibly the majority of them do this.

The forms intermediate between this and the segmentation stage appear in the fresh blood simply as larger parasites, which are readily found on account of the reddish-brown pigment granules that they contain. These granules begin to appear several<sup>1</sup> hours after the organism has infected the red blood cell. At this time the organism is usually actively ameboid and the granules have a lively dancing motion, due to protoplasmic currents in the parasite. The infected corpuscle is swollen and paler. In the estivo-autumnal form the infected red blood cells are smaller than normal.

When the parasite has approached nearly to its full growth, it occupies the greater portion of the corpuscle, which is now more difficult to make out. The pigment is still more evident, so that this form is therefore most readily found. At this stage ameboid movements are not so active. When full growth is reached, segmentation occurs. The forms up to the period of segmentation are called schizonts.

The morphological changes which have been going on in the parasite preparatory to segmentation are best studied in properly stained smear preparations. In the living organism they become presently sufficiently distinct to be followed; the pigment gathers more or less centrally into a compact mass, and a peripheral notching indicates that the parasite is preparing to divide into a number of segments called merozoites; the number of these segments varies in the different species. (See table.) Suddenly the segments separate as small spheroidal

<sup>1</sup> See table for number of hours in each species.



TABLE SHOWING CHIEF DIFFERENCES BETWEEN THE SPECIES OF MALARIAL ORGANISMS (GENUS *HÆMAMEBA*) FOUND IN MAN.

Name of organism.	Size of parasite up to segmentation (schizont).	Motion of young schizont in corpuscle.	Time of appearance of melanin granules and their arrangement.	Shape of segmenting parasite; number of segments, and site of segmentation.	Asexual cycle complete in	Sexual forms.	Incubation period.	Effect on human host tissues.	Remarks.
<i>H. vivax</i> (parasite of tertian fever).	1 $\mu$ to slightly larger than normal red blood cell (may occasionally be almost twice size)	Markedly active	6 hours; first scattered, then gathered in centre; finely granular; actively dancing	Irregular mulberry; 12-24 (average 16); peripheral circulation	48 hours	Gametocytes spherical; no crescents; male 1 size of female, which is 1 1/4 times size of red blood cell	About 14 days	Pale, granular; slightly enlarged red blood cells; finely granular pigment formed from metamorphosed hemoglobin	Double infection may cause a paroxysm every day; thus giving clinically a quotidian type of fever.
<i>H. malaris</i> (parasite of quartan fever).	1 $\mu$ to little less than size of red blood cell	Not very active	Within a few hours; collected in zone on periphery; coarsely granular; slight dancing	Regular delay in shape; 6 to 10 (average 8); peripheral circulation	72 hours	Gametocytes spherical; fewer than in vivax; about size of red blood cell; no crescents	About 3 weeks	Red blood cells may be slightly shrunken	Triple infection may cause a paroxysm every day; thus giving clinically a quotidian type of fever.
<i>H. falciparum</i> (parasite of estivo-autumnal or pernicious fever).	Smaller than others, from very small 1/2 diameter corpuscle	Active; slightly less so than tertian type	Within 24 hours; small amount; 2 or 3 coarse granules usually central; non-motile or sluggish	More or less symmetrical; delay; 10 to 32 very small; chiefly in bone, marrow and viscera	24 to 48 hours	Gametocytes crescentic, short and plump; 1/2 size of red cell	About 10 days	Red blood cells unstained, greenish, shriveled (crenated) and darkened; stained (Giemsa) salmon color.	

bodies, the young parasites. A corpuscular remnant and the pigment float away and are ultimately ingested by phagocytic cells. The young parasites attach themselves to red corpuscles as before and the human cycle is repeated.

In a suitably stained preparation the young parasite (see Plate VII), appears to be a disk consisting of a central pale, unstained area, known as the achromatic zone, and of a basic (blue) periphery, the body, including a metachromatically stained, rounded, compact (red) chromatin mass, the nucleus, which tends to give the parasite the form of a signet ring.

Later stages up to a certain number of hours show simply changes in size and outline of the body. The nucleus then divides by simple mitosis. Later it breaks up by amitotic division into an increasing number of small masses. By the time the chromatin division is completed the chromatin masses will have assumed a rounded form, and will be seen to exhibit ultimately the same strong affinity for certain dyes which is seen in the compact chromatin body of the young ring-like form. At this stage the heretofore scattered pigment appears in one clump. Good technic will always show a corpuscular remnant even at this time. The achromatic zone mentioned will be seen to develop with the chromatin, and when the next step, namely, the division of the body of the parasite, is seen to be completed, there will be as many achromatic bodies as there are chromatin bodies, each division having a share of the basic mother-body, each representing the young parasite (merozoite).

A certain number of the full-grown parasites do not segment and these are the forms which commence the life cycle in the mosquito. These forms grow to produce the sexual forms, the macrogametocyte, or female organism, and the microgametocyte, or male organism. When mature these forms are generally larger than the mature schizont of the same species, the female organism being usually larger than the male and containing more food granules and a smaller nucleus. In the estivo-autumnal forms they are crescentic in shape, while in the other species they are spherical. In the circulating blood of human beings they show no further changes except to become freed from the corpuscle; but when the blood containing them is withdrawn and exposed for a short time to the air, an interesting series of changes in the microgametocyte is observed. The crescentic bodies are transformed into spherical bodies; the pigment of the microgametocytes becomes actively motile, due to internal agitation of the chromatin fibrils, which presently emerge as flagella-like appendages. Their movements are very rapid, causing corpuscles to be knocked about, and finally they become detached as the microgametes, or male elements, and go in search of the female element. In the withdrawn blood of birds, one may actually observe the process of conjugation in slide preparations even without the aid of a moist chamber and heat. This transformation of male bodies never occurs in the human blood. It will be seen that it belongs to the sexual cycle which occurs in the stomach of the mosquito.

**The Sexual Cycle (Sporogony) Developing in the Mosquito.**—The common mosquito, often day-flying, belongs to the genus *Culex*; it cannot carry human malaria. It is easily distinguished from its night-flying or dusk-flying relatives, *Anopheles* (the malaria-carrying mosquitoes comprise about eight genera of the subfamily anophelinæ), by its assuming a different posture on the perpendicular wall. While the *Culex* holds the body more or less parallel with the surface, the body of the *Anopheles* stands off at a marked angle. Other differential points are the following (see Figs. 184-193):

Wings of *Culex* are unspotted; those of *Anopheles* are spotted (except in one rare species).

The proboscis of *Anopheles* points toward the resting surface, while that of *Culex* does not do so.

*Anopheles* species bite usually in the early evening, while those of *Culex* bite almost at any hour of the day.

The male mosquito is readily told from the female by its plumed antennæ, those of the female being inconspicuous.

The eggs and the larvæ of the two genera are quite distinct, as may be readily seen by glancing at Figs. 184 and 185. The anopheles mosquitoes breed in practically any kind of a collection of water, though some species prefer slow-running water to quiet pools. The best known domestic carriers are usually found in barrels and cisterns.

If an ordinary mosquito (*Culex*) is allowed to imbibe the blood of a malarial patient whose blood shows gametocytes there will be simply a digestion of such blood in the mosquito, and no development of the malarial organisms results. If, however, certain species of *Anopheles* ingest such blood, immediate changes follow. It should be remembered that only female mosquitoes are blood sucking; hence they alone can be responsible for the spreading of the disease. It should also be remembered that if the blood imbibed by the anopheles does not contain gametocytes, though it may contain earlier stages of the malarial organisms, no amount of such blood can cause general infection of the mosquito. The sexual cycle is similar in all species of the parasite.

The flagellation of the male parasite described above will promptly take place in the stomach of the anopheles, 4 to 8 microgametes being formed; these conjugate with the female element (Plate VII) in a manner comparable to the impregnation of the ovum of higher animals by spermatozooids. The macrogametocyte becomes a macrogamete by the formation of a reduction nucleus which is thrown out of the organism.

The product of conjugation, the oökinet (zygote), remains for a number of hours in the juices of the chyme stomach, changing gradually from a spherical, immobile body into an elongated motile wormlet (Plate VII, Sexual Forms, 1 H). This penetrates the epithelial lining of the stomach and rests in the tunica elasticsearchomuscularis (Plate VII, Sexual Forms, Fig. 2); here it changes into an oval then into a round body, which grows in the course of the next few days enormously, forming a cyst which projects into the body cavity. Meanwhile the

FIG. 184



FIG. 185



FIG. 186

FIG. 187



FIG. 188



FIG. 189



FIG. 190



FIG. 191



FIG. 192



FIG. 193



Chief comparative characteristics of *Culex* and *Anopheles*. (From Kolle and Hetsch.) Egg of *Culex*, Fig. 184, laid together in "small boat," those of *Anopheles*, Fig. 185, separate and rounded. Larva of *C.*, Fig. 186, hangs nearly at right angles to water surface, those of *A.*, Fig. 187, are parallel to surface. Body of *C.*, Fig. 188, when resting is held parallel to wall in a curved position, that of *A.*, Fig. 189, stands at an angle of about  $45^\circ$  and is straight; wings of *C.*, Fig. 190, are generally not spotted, those of *A.*, Fig. 191, are spotted. In *C.* the palpi, Fig. 192, of the female are very short, of the male are longer than the proboscis; in *A.*, Fig. 193, the proboscis of both sexes are about of equal length.

chromatin will have become very active. It will have divided into numerous nuclei, which become arranged around inactive portions, and filamentous sporozoites develop from this chromatin and surrounding protoplasm (Plate VII, Sexual Forms, Fig. 2). These sporozoites ultimately fill the cysts, which rupture, setting them free into the cavity of the mosquito's body; they then are carried by the lymph to all parts of the body of the mosquito and thus reach a glandular structure in the thoracic cavity of the insect, the so-called salivary gland (poison gland), in which they accumulate in large numbers. This gland is in immediate connection with the biting and sucking apparatus. If, now, such an infected mosquito "bites" a human being, the lubricating fluid of the puncturing apparatus will carry sporozoites into the latter's blood and the human cycle begins. The stages of development in the mosquito require from seven to ten days, but only when the temperature is favorable.

**Cultivation.**—Bass and Johns announced in 1911 that they had succeeded, by a rather complicated method, in obtaining a certain amount of development of *H. Vivax* in the test-tube (see p. 106 for details of method). Essentially, defibrinated malarial blood is used, to which is added  $\frac{1}{2}$  per cent. dextrose. Bass and Johns state that the layer of serum about the sedimented red blood cells must not be too deep ( $\frac{1}{2}$  inch) and that the leukocytes must be removed if more than one generation of the parasite is wished. The Thompsons (1913) state that they have gotten several generations by less attention to these details.

They draw the malarial blood into the sterile test-tube (10 c.c.) in which there is a thick wire, and 0.1 c.c. of a 50 per cent. aqueous solution of glucose. The blood is defibrinated by gently stirring the wire for five minutes. The wire and clot are removed, and the blood is poured into smaller tubes, 1 inch in each. Rubber caps are placed over cotton plugs. These tubes are kept at 37° to 44° C. The corpuscles settle slowly, leaving about  $\frac{1}{2}$  inch clear serum. They have found it unnecessary to remove the leukocytes by centrifuging. They state that the malarial organisms are not destroyed by the leukocytes in the tube but by the changes in the serum. Their organisms grow through the whole depth of the layer of red blood cells.

**Effect on Man (Pathogenesis).**—As the organism grows at the expense of the red blood cells the principal change is in the blood. Melanemia, or the formation of pigment granules from the destroyed red blood cells, is one of the most characteristic features of malaria. As the disease progresses the red corpuscles show varying changes in form and hemoglobin content, not only the infected corpuscles, but others as well, thus showing that the organism produces either primarily or secondarily some toxic substances. The pigment occurs in two forms, melanin and hemosiderin. The second only gives the reaction for iron and is found in the internal organs, while the first is found everywhere in the circulating blood. The pigment is taken up by the leukocytes. There is usually a definite reduction of both red and white blood corpuscles, which is more marked in tertian and quartan malaria than in estivo-autumnal. There is a relative increase in the number of mononuclear leukocytes. The spleen shows marked hyperplastic inflammation and pigmentation. In intense estivo-autumnal cases the capillaries of brain and other organs may be filled with the parasites. We have observed parasites also in the large nerve cells of the brain.

**Toxic Production.**—The relationship between segmentation and paroxysm is always noted in tertian cases, and it is reasonable to suppose that the occurrence of the paroxysm is referable entirely to the liberation of toxic substances resulting from metabolic activity of the parasite within the corpuscle. That there should be a toxic product seems highly probable, and its amount must be considered in heavy infections. Cases showing an infection of 1 to 5 per cent. of all corpuscles are not infrequent; the destruction of from 50,000 to 200,000 or more corpuscles per cubic millimeter of blood leads to the rapid deglobularization of the blood; hence the deficiency in numbers; add to this the effects of the metabolic products, and little is left to the imagination to explain the pronounced anemia.

**Immunity** from malaria appears to exist as natural and acquired immunity.

**Prophylaxis.**—The fact that, with the extermination of the malaria-carrying mosquitoes, malarial fevers in man would be made impossible, remains established; the parasite must have its chance of rejuvenescence in the mosquito's stomach.

The various methods of extermination are fully described in books which go minutely into the subject. The method of giving small doses of quinine to human beings exposed to *Anopheles*, and of thus getting rid of the organism itself within man, should be considered. In hot climates especially, where it is practically impossible totally to destroy the breeding places of the mosquitoes by drainage or oiling, this method is especially serviceable. In these countries, too, the use of adequate screening is of marked value.

**Points of Diagnosis.**—By a study of the parasite taken from the circulating blood the examiner should be able to tell not only the species present, but also the progress the disease is making. Malarial parasites can always readily be found in recent primary infections, and it is usually only in old cases that the search becomes difficult; one is, however, generally rewarded by finding them if one looks long enough for them.

A helpful sign is the finding of pigment in mononuclear leukocytes, which are seen about the time of a chill or of the period symptomatically corresponding to it. Free pigment cannot be used as a means of diagnosis, as it may be impossible to tell it from dirt or dust. In a primary infection of *long standing* the gametocytes may be found, and in relapses and in those cases treated by quinine, many atypical forms appear. A small dose of quinine may drive all parasites except the sexual forms out of the peripheral circulation; at all events the finding of them becomes, in the absence of gametocytes, a matter of time and experience, especially also as they may be much altered in appearance. The part most and first affected is the blue-staining body; later follow eccentricities of the chromatin, such as multiple bodies, and dwarfing, just such changes as might have occurred in time, if the body had been allowed to combat the parasite without the aid of drugs. In both cases the fever curve becomes atypical. It

should be remembered that there is no quotidian form originating in this country. Quotidian paroxysms occurring here are either a double tertian, or a triple quartan infection. The notion that the parasites can be found only at the time of the paroxysm is still in the minds of many; it is erroneous. The gametocytes are quite resistant to quinine and other drugs, and it appears as if cases in which these forms are seen are much more prone to relapse than promptly treated recent primary infections. The macrogametocytes may remain quiescent for years in the blood, and then under certain conditions, probably through parthenogenesis, may again begin to develop and multiply, thus bringing about relapses.

In the estivo-autumnal forms the crescentic gametocytes are generally few, but at times large numbers of them develop. Of course they are absolutely characteristic. The young parasites are more or less characteristic in stained preparations (Plate VII). There may be as many as seven parasites in one corpuscle. Later the few heavy pigment granules are characteristic.

In fatal cases the formation of crescents may not take place; the blood infection with young parasites is then enormous, every field of the microscope showing numbers of them.

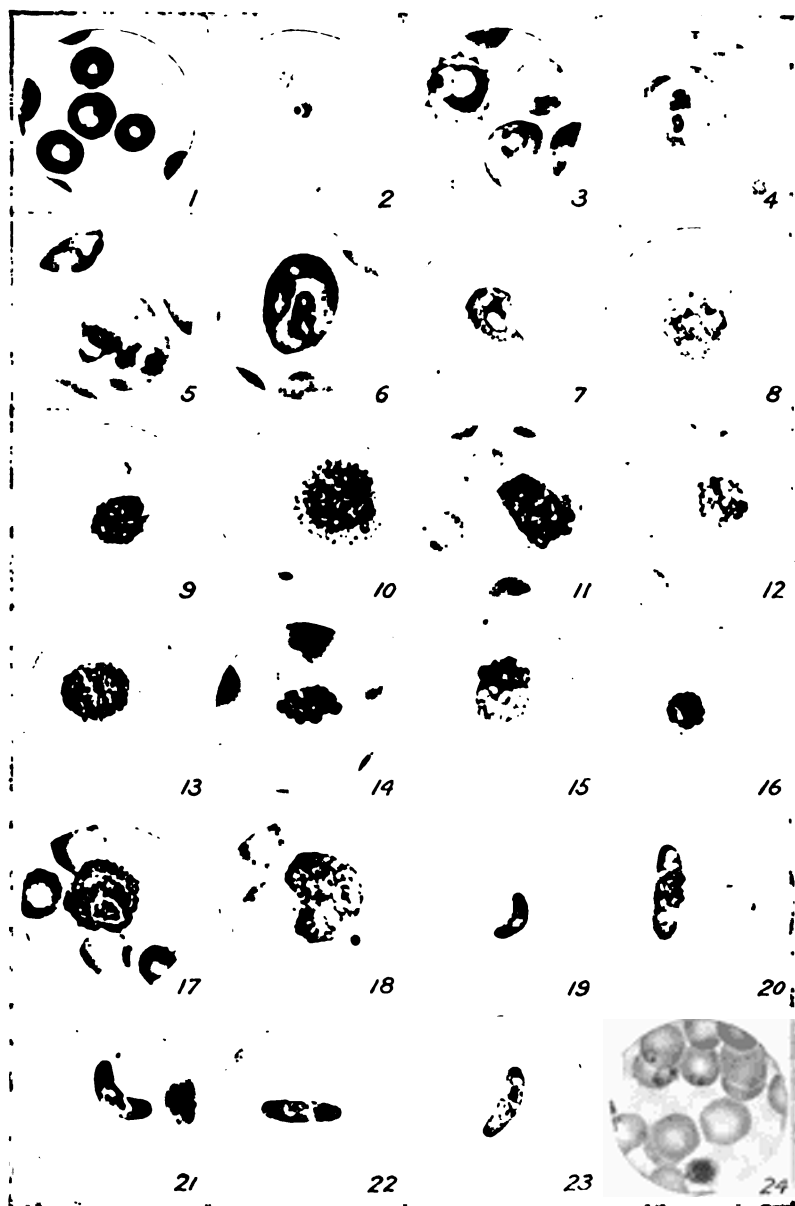
#### DESCRIPTION OF PLATE VIII.

(After Goldhorn.)

1. Typical young tertian form; the corpuscle shows incipient degeneration; corpuscle to left above shows a blood platelet.
2. Abnormal young form, showing small accessory chromatin body.
3. Two parasites; one normal young form; the second a large form in crenated corpuscle is an unusual abnormal form with very large achromatic area.
- 4, 5, 6. Estivo-autumnal parasites; single, double, and triple infection; central elongated chromatin bodies. These forms are about the largest usually seen in the peripheral blood; no degeneration of corpuscle.
7. Tertian parasite, about ten hours old; marked degeneration of corpuscle.
8. Double infection of a corpuscle in tertian fever; marked degeneration of corpuscle.
- 9, 10, 11. Large tertian parasites showing division of chromatin previous to segmentation.
- 12 and 14. Complete segmentation of tertian parasite.
13. Double infection of corpuscle, one parasite reaching maturity, but showing unusually small segments; the second one atrophied.
15. Tertian parasite, old case; while the parasite is only half-grown, the chromatin has split into several compact masses. Degeneration of infected corpuscle.
16. Dwarfed tertian parasite, smaller than a red corpuscle, but showing five compact chromatin bodies; resemblance to quartan rosette.
17. Microgametocyte of tertian malaria; prominence of blackish pigment surrounding a large achromatic zone in which the microgametes lie coiled up.
18. Tertian macrogametocyte.
- 19 to 23. Crescentic bodies of estivo-autumnal malaria.
19. Typical gametocyte; pigment surrounding achromatic area; no chromatin shown; the "bib" is present. (Male?)
20. Semiovoid gametocyte. (Female?)
21. Pigment removed. Elliptical achromatic area in which the microgametes are seen.
- 22 and 23. Pigment removed; chromatin more compact; possibly female elements.
24. From a case of pernicious malaria with rich infection; only hyaline forms in peripheral blood. Below, a large blood platelet.

NOTE.—As the amplification is not uniform, a comparison of the parasites with the blood corpuscles shown should be made in order to have a correct conception of their size.

# PLATE VIII



Photographs of Tertian and Estivo-autumnal Malarial  
Parasites in Different Stages of Development.  
(Goldhorn.)





In the study of estivo-autumnal fever, as well as in that of the other forms, it is to be remembered that crescents when found indicate that the disease is of some standing, for such sexual forms are not formed until the asexual propagation is waning. The recognition of these ovoidal and crescentic bodies is easy. But as there are no readily discoverable pigmented forms in the peripheral blood in the early stages, it is necessary to be thoroughly familiar with the young estivo-autumnal forms. Polychrome staining for them cannot be too much recommended, as there is little that is characteristic about them when they have been stained with methylene blue alone. Many a serious error has been made by adhering to the antiquated idea that parasites should be looked for in the fresh blood, as these young, non-pigmented, so-called hyaline forms cannot be readily recognized by the inexperienced, while it is an easy matter to know and classify them when properly stained.

The recognition of the quartan parasite in its early stages in the fresh blood is not as difficult as that of the tertian form, because the outline is more distinct; but in stained preparations it is often indistinguishable from the latter. The living ameboid young form or schizont is more refractive than the young living tertian schizont, more like the estivo-autumnal form, and it is just as sluggish in its movements. Then, too, the corpuscle is often shrunken and looks as if it contained more hemoglobin than in the case of infection with the tertian parasite.

The growing parasite rapidly becomes pigmented, but it shows fewer, larger, less motile pigment granules than the corresponding tertian one; moreover, the pigment is arranged around the periphery of the organism, while in the tertian form it is distributed throughout the protoplasm. The quartan parasite is apt to form a band across the infected corpuscle. Segments are few in number, as a rule, and the parasite remains dwarfed while the infected red blood cells are normal in size. The segments are generally arranged symmetrically around the central pigment, giving the so-called daisy or marguerite appearance to the parasite at this stage (Plate VII).

In tertian fever the granular degeneration which the infected corpuscles early undergo is diagnostic. In the first few hours it resembles the ordinary granular stroma degeneration with basic affinity, while it is later seen that the affinity of the then more numerous granules is more acid, or, at least, the staining is no longer orthochromatic, the blue being superimposed by a red; in other words, these granules stain later metachromatically. The greater the loss or transformation of the hemoglobin the greater the number of granules. This holds good only for tertian parasites, the estivo-autumnal variety causing practically no appreciable change, though the same technic is used.

**Malarial-like Parasites in Other Animals.**—Two genera of protozoa closely related to the malarial organisms have been found in birds: (1) the proteosoma or hemoproteus; (2) the halteridium; both found

in owls (*Hæmoproteus noctuæ* Celli and Sanfelice). Points in their life history have been brought out by various observers, especially by Ross and by MacCallum. The complete life cycle of both forms, as worked out by Schaudinn, is considered by him and his followers to be of fundamental importance to the understanding of the relationship of blood parasites. Schaudinn states that these organisms pass through a flagellate stage in the intestinal tract of the common mosquito (*Culex pipiens*) which has previously fed on owls infected with the intracellular organisms (halteridium and hemoproteus). Novy considers that this mosquito flagellate stage of Schaudinn is simply a growth of trypanosomes in the mosquito's intestinal tract which are normally found there, and that Schaudinn did not sufficiently control his work to warrant his conclusions.

Malarial-like organisms have been found also in monkeys, cattle, dogs, and frogs, but they have been little studied.

An interesting article by Bernberg-Gossler on malarial organisms in monkeys was published in 1909. In it the author describes a binucleate phase of these plasmodia and agrees with Hartmann in his recent classification of these organisms.

**Blackwater Fever.**—This is a condition which occurs frequently, especially in Europeans in tropical countries. Its etiology has been the subject of much discussion. The chief symptoms are fever, hemoglobinuria, delirium, and collapse. It frequently ends in coma and death. A few consider it a disease entity, but the majority of observers are inclined to believe it the terminal stage of a severe malarial infection. It is frequently associated with the demonstration of the malarial organism, but they are not always found. It may be that the invasion of the ganglion cells of the brain by the malarial organisms are the chief cause of the symptoms, aided in certain cases by the lysemia noted by Christophers and Bentley.

### GENUS BABESIA (PIROPLASMA).

It was not until 1888 that there was a hint as to the real nature of the actual cause of "Texas fever" (bovine malaria, tick fever, hemoglobinuria) and allied diseases which attack field cattle in many parts of the world. Then Babes described inclusions in red blood cells in Roumanian cattle sick with the disease, though he did not decide upon the nature of the organism. No new studies were reported until 1893, when Theobald Smith and Kilborne gave such a complete description of this disease and its cause as occurring in Texas cattle that little concerning it has since been discovered.

These authors describe as the cause of Texas fever, pigment-free ameboid parasites appearing in various forms within the red blood cells of infected animals. The organisms may be irregularly round and lie singly or they may be in pear-shaped twos, united by a fine line of protoplasm.

Because of these double pear-shaped forms Smith and Kilborne named the organism *Pyrosoma bigeminum*<sup>1</sup> and they placed it provisionally among the hemosporidia. These authors also showed that the contagion was carried by a tick (see p. 550). Their work has been corroborated by many investigators in different parts of the world. Hartmann places this genus in his new order Binucleata, and he considers it an important form for showing the relationship of the endocellular blood parasites to the flagellates. Schaudinn, in 1904, was the first to call attention to the occurrence of nuclear dimorphism in *B. canis* and *bovis*, and Luhe, Nuttall and Graham-Smith, Breinl and Hindle, and others have confirmed this observation. The second nuclear mass is generally in the form of a small granule similar to the blepharoplast of undoubted flagellates.

**Morphology of the Parasite.**—In the examination under 1000 diameters of fresh blood of sick cattle, according to Smith and Kilborne, are seen, in the red blood cells, double pear-shaped forms and single rounded or more or less irregular forms. The size varies, though generally it is the same among the bodies in the same red blood cell. The average size is  $2\mu$  to  $4\mu$  long and  $1\frac{1}{2}\mu$  to  $2\mu$  wide. The pointed ends of the double form are in apposition and generally touch, though in unstained specimens a connection between them cannot be seen. The axis forms either a straight line or an angle. The protoplasm has a pale, non-granular appearance, and is sharply separated from the protoplasm of the including red blood cell. The small forms are generally fully homogeneous, whereas the larger ones often contain in the rounded ends a large rounded body,  $0.1\mu$  to  $0.2\mu$  in size, which is very glistening and takes a darker stain. Within the largest forms in the centre of the thick end is a large round or oval body,  $0.5\mu$  to  $1\mu$ , which sometimes shows ameboid motions. Piana and Galli-Valerio (1895 and 1896) and other observers have since described definite ameboid motion of the whole parasite. The motion of the whole parasite on the warm stage is not produced by the formation of distinct pseudopodia, but by a constant change of the boundary. The changes can succeed each other so quickly that it is scarcely possible to follow them with the eye. The motion may persist for hours. The single ones show motion, while the double ones remain unchanged.

The parasites take most basic aniline stains well. The Romanowsky method or its modifications gives the best results (Plate IV, Fig. III, B). Stained by this method the smallest forms appear as tiny rings, about one-sixth the diameter of the red blood cell. A part of the rim takes the red nuclear stain, the rest is blue. In the large mature pear-shaped organisms a loose mass of chromatin is at the rounded end and a dense, compact mass is situated nearer the pointed end. These mature, pear-shaped forms, Nuttall states, are the mark of distinction between *Piroplasma* (Babesia) and other intracorpuseular blood parasites. These pyriform bodies are generally present in pairs, and

<sup>1</sup>The generic name *Pyrosoma*, already in use for a well-known Ascidian genus, was altered to *Piroplasma* by Patton in 1895. In the meantime Starcovič (1893) had given the name *Babesia bovis* to the form described by Babes; and as this form seems to be identical with that described by Smith and Kilborne the correct name of the genus should be *Babesia* while the species parasitic in cattle should be called *Babesia bigemina*.

occasionally, in the acute form of the disease, sixteen pairs may be seen in a single blood cell.

The number of red cells infected is about 1 per cent. of the whole. If the number increases to 5 per cent. or 10 per cent., it generally means the death of the animal. The parasites quickly disappear from the blood after the disappearance of the fever. In fatal cases many parasites are found in the red blood cells of the internal organs. They vary in number according to the stage at which death occurs, are most abundant in the kidneys (50 to 80 per cent. of all red corpuscles infected), and are found in fewer numbers in the liver, spleen, and other internal organs.

R. Koch has described a bacillar form which he found in large numbers in red blood cells of acute fatal cases in East Africa. Between these and the pear-shaped forms he found all grades. This variety is probably a distinct species.

Flagella-like appendages in *Babesia* have been described by several observers as occurring in the blood in mammals. More frequently they have been seen in the tick and in attempted cultures. They have been interpreted by some (Hartmann, Calkins) as possible microgametes, by others (Breinl and Hindle) as true flagella, and by others (most observers) as fine pseudopodia.

Smith and Kilborne showed that the infection is caused by a species of tick, *Margaropus annulatus* Say (*Boöphilus bovis*) (Fig. 194), and Kossel gives *Ixodes redivius* as the tick causing transmission of the germ in the hemoglobinuria of Finland cattle.



FIG. 194.—No. 1, Texas fever tick, *Margaropus annulatus* (*Boöphilus bovis*).  $\times 15.4$   
No. 2, natural size. (Mohler.)

The ticks feeding upon the blood of cattle and other mammals become sexually mature at their last moult. They then pair, and the fertilized females, after gorging themselves with the blood of their host, drop to the ground. Each female then lays about 2000 eggs, and within the shell of each egg a large quantity of blood is deposited to serve as food for the developing embryo. The female then shrivels up, becoming a lifeless skin. The newly hatched larvæ containing in their abdomens some of the mother-blood, crawl about until they either die from starvation or have the opportunity of passing to the skin of a fresh host. If the mother-tick has drawn its supply of blood from cattle infected with piroplasma, her larvæ are born infected with

the parasite and become the means of disseminating the disease further. This mode of dissemination explains the long incubation period of the disease (forty-five to sixty days—thirty days for the development of the larvæ and the remainder for the development of the parasite within the host). It is possible that the tick embryo acquires the infection secondarily from the blood it absorbs in the egg, and that the parasites do not pass through the ovum itself as in *Nosema bombycis*. This species of tick *M. annulatus* has been found also on sheep and ponies.

So far it has not been possible experimentally to inoculate animals other than cattle with these parasites. Calves withstand the infection better than older animals and a certain degree of immunity is reached in some of the older cattle in infected districts. The piroplasmata taken in by such animals may remain as harmless parasites for some time. If, however, such cattle are weakened from any cause, their resistance to the organism may be lowered and they may therefore pass through a more or less severe attack of the disease.

**Symptoms of the Disease.**—Fever ( $40^{\circ}$  to  $42^{\circ}$  C.), anorexia, weakness, increased pulse and respiration, decreased secretion of milk, hemoglobinuria at the height of the fever, causing the urine to appear dark red like port wine or darker. The urine may contain albumin even if the hemoglobinuria is absent, but there are no red blood cells present, the color being due to the coloring matter of the blood only. There is icterus of the mucous membranes if much blood is destroyed.

**The prognosis** varies in different epidemics from 20 to 60 per cent. Death may occur in three to five days after first symptoms appear. Recovery is indicated by a gradual fall of the fever.

**Treatment.**—Quinine in large doses seems to have helped in some epidemics. Nuttall, Graham-Smith, and Hadwen have reported curative effects from trypanblau in both canine and bovine babesiosis (Piroplasmosis).

**Prophylaxis.**—Stalled cattle are not infected, but it is impracticable to keep large herds of cattle stalled. If the cattle are kept from infected fields for one or two years and other animals (horses and mules) are allowed to feed there the ticks may disappear. The burning of the field for one season may have a good effect. If animals cannot be taken from infected fields such fields should be enclosed.

Ticks on animals may be killed by allowing the cattle to pass through an oil bath (paraffin, cottonseed oil, etc.), whereupon the ticks die from suffocation. The bath should be repeated after a week in order to kill any larvæ which may have developed. All animals sent from infected regions should receive this treatment. Animals apparently healthy before the treatment, after the disturbing influence of the bath often develop the disease in an acute form and die.

Certain birds in Australia seem to feed on the ticks, therefore such birds might be propagated.

Various attempts have been made to give protection by the inoculation of fresh (not older than two or three days) blood from slightly infected animals. Some partial results have been reported, especially when the inoculations were made during the cold months. In Australia the inoculation of defibrinated blood from animals which have just recovered from the infection, but whose blood still contains some parasites, has been tried. So far no absolute protection has been produced, neither does the parasite-free serum of animals which have entirely recovered from the disease seem to contain protective qualities.

**Cultivation.**—Thompson and Fantham have reported successful development in the test-tube after the method of Bass and Johns for malaria.

Nuttall and Graham-Smith report a study of canine piroplasmosis, and have drawn a cycle showing the usual mode of multiplication in the circulating blood. They consider *B. canis* a species distinct from *B. bovis* and *B. pitheci* (found by Ross, in 1905, in blood of a species of cercopithecus) though no morphological differences are given.

Christophers has described probable sexual stages of development in the tick *R. sanguineus*, so that he has drawn a complete life cycle of the organism.

**Other Blood Organisms.**—Blood organisms similar to those described in the hemoglobinuria of cattle have been found in cases of red water fever of cattle in England. They also occur in monkeys, dogs, sheep, horses, and pigeons. Nocard and Motas, who have made an extensive study of these parasites in the malignant jaundice (hemoglobinuria, malaria, or biliary fever) of dogs, state that though the parasites are morphologically similar to those infecting cattle, yet it is impossible to infect cattle or any other animal tried with them. They must therefore be considered a physiological variety.

Strong and his collaborators reported that in an extensive study of *oroya fever*, a tropical disease, they had determined the cause to be a Babesia-like organism which they called *Bartonia bacilliformis*.

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## CHAPTER XLIV.

### SMALLPOX (VARIOLA) AND ALLIED DISEASES.

**Introduction.**—The diseases smallpox, cow-pox, vaccinia, horse-pox, sheep-pox, if not identical, are closely allied. Indeed, the following facts seem to prove that at least cow-pox and variola are very closely related, if not essentially the same disease: *First*, smallpox virus inoculated into calves produces, after passage through several animals, an affection exactly similar to cow-pox. The successful inoculation of the first series of cattle from smallpox is a matter of great difficulty, but so many experimenters have asserted that this has been done that there seems to be no doubt as to its truth. In our laboratory not one of many attempts to accomplish it has been successful. *Second*, both when occurring in nature and when produced by experiment the lesions of the two diseases are similar. *Third*, monkeys have been successfully protected against either disease by previous inoculation of the other; also, observations go to show that human beings inoculated with cow-pox vaccine are not susceptible to inoculation with smallpox virus, and that those who have within a varied time passed through an attack of smallpox cannot be inoculated successfully with cow-pox vaccine. These facts seem positively to prove that the two diseases are produced by organisms originally identical, one being modified by its transmission through cattle, the other through human beings.

Variola is perhaps the most regularly characteristic of the diseases of man. It is highly infectious and is controlled only by vaccination. Notwithstanding the fact that we know definitely the exact site of the infective agent in this disease and that certain experimental animals are susceptible to inoculation of the material containing the infective agent, most investigators are still undecided in regard to the nature of the chief exciting factor. A few, however, claim that certain bodies found chiefly in the epithelial cells of the skin and mucous membranes in the specific lesions are protozoa causing the disease.

**Definition.**—Smallpox (Synonyms: Variola, la variola, Blattern, Pocken, Vajuola) is an acute infectious disease characterized by an epidermic eruption of vesicles and pustules which, upon healing, produce cicatrices of varying extent and depth.

**Historical Note.**—The first undoubted description of the disease was given by Rhazes in the tenth century, but it is evident that he did not consider it a new disease. To trace its original home seems to be impossible. It may have developed first in certain regions in Asia and Central Africa where it is at present endemic and is said to be uncontrolled by vaccination. Many outbreaks of the disease in the United States can be traced directly to the importation of African negroes.



The disease, carried by the intercommunication, principally of war and commerce, was widespread when Edward Jenner showed conclusively in 1798 that vaccination with cow-pox afforded protection. Now the few cases of variola that occur are seen in those who, through neglect or ignorance (sometimes wilful), have not been vaccinated.

**Etiology of Variola and Cow-pox.**—It has been repeatedly shown that no bacteria similar to any of the known forms have a causal relation to these diseases. In our own laboratory we are able, by the inoculating of rabbits' skins, to produce extremely active vaccine virus in large quantities, absolutely free from microorganisms which grow under the conditions of our present methods of bacterial cultivation. Such pure active vaccine, when emulsified in equal parts of glycerin and water and filtered through two or three thicknesses of the finest filter paper, gives a slightly opalescent filtrate, which in the hanging drop under high magnification shows many very tiny granules with an occasional larger one, and in smears shows no formed elements giving characteristic stains. This filtrate, from which no growth was obtained on artificial culture media, when rubbed over a freshly shaved rabbit's skin after the method of Calmette and Guérin, or when used to vaccinate human beings, gives an abundant typical reaction.

These facts show that some, at least, of the infective forms cannot as yet be made to grow outside of the body, that such forms are very minute, and that they do not stain characteristically with our usual methods of staining. In a few experiments we were unable to filter the virus through a Berkefeld filter under forty pounds' pressure, but this may have been due to the fact that we did not dilute our virus sufficiently. Since then Bertarelli and a few others have reported that the virus is slightly filtrable under pressure. Park and Wilson, however, on later experiments (1913) still obtained only negative results.

Steinhardt, Israeli, and Lambert (1913) report that evidence of multiplication of germs may be obtained by growing the virus on living tissue *in vitro*.

Since Guarnièri, in 1892, claimed that certain inclusions present in the epithelial cells of the lesions of smallpox in a rabbit's cornea (Fig. 195) were parasites, much attention has been given to the study of these bodies, commonly known as "vaccine bodies," yet opinions still differ as to their nature. Among the more important studies of these bodies are those, on the one hand, by Councilman and his associates, who believe them to be protozoa, and, on the other, by Ewing, who believes that all of the forms so far described are degeneration products, some specific, others not.

Calkins, working with Councilman, thinks that his original tentative cycle is too elaborate. He still firmly believes that the bodies are protozoa, but that they belong among the rhizopoda and not among the microsporidia where he first placed them.

Prowazek and others believe that the organisms of this group of diseases, as well as of rabies, scarlet fever, trachoma, and a few others, are all minute coccus-

like forms which have the power of producing an envelope from the host cell substance, such envelope with its contained organism constituting the specific body which others have called a protozoön. Prowazek calls the group *Chlamydozoa* and says they probably stand between the bacteria and the protozoa in systematic classification. From our studies on this whole group of diseases we have come to the conclusion that there is no close relationship between the trachoma bodies and the intracellular bodies of rabies, smallpox and scarlet fever (see pp. 415 and 561).

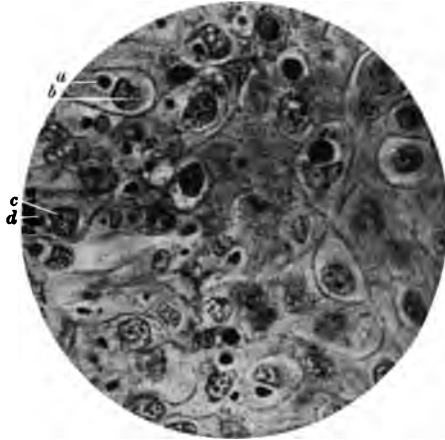


FIG. 195.—Epithelial cells of a rabbit's cornea, containing many "vaccine bodies." Tissue fixed three days after inoculation with smallpox virus. *a* and *d*, vaccine bodies; *b* and *c*, nuclei.  $\times 1500$  diameters.

In our own work on sections, which has extended irregularly over a period of several years, we have gotten results which are somewhat confusing, principally because of the non-uniformity of the appearances of these bodies, both by different methods of demonstration and by the same methods at different times. There is no doubt that, whatever the nature of the bodies, they are easily affected by methods used for fixing, hardening, and staining them. This accounts in part for the varied results reported. However, in the most perfectly prepared specimens, judged according to the appearance of the red blood cells, leukocytes, and tissue cells at a distance from the lesions, we have found that the vaccine bodies, especially in corneal infection, show a more or less constant series of changes, somewhat similar to those described by Calkins in his "gemmule formation" and by Tyzzer in his development of the vaccine bodies.

Our best results on corneas have been obtained with the following technic: Fix in Zenker's fluid for from four to eight hours; wash in running water overnight; place in 95 per cent. alcohol (changing in two hours to fresh) for twenty-four hours, then in absolute alcohol for twenty-four hours. Imbed in paraffin. The cuts should be from  $3\mu$  to  $5\mu$  thick. Stain with (1) eosin and methylene blue (Mallory)—eosin half an hour, methylene blue two minutes; (2) Heidenhain's iron hematoxylin; (3) Borrel modified by Calkins.

The vaccine bodies may be studied for a short time in the living cornea by rapidly excising an inoculated cornea, spreading it on a shallow agar plate and dropping a thin cover-glass over it. The structured bodies are very clearly differentiated from the rest of the cell contents, and interesting changes have been observed in them. Too little work has been done, however, by this method, to draw any further conclusions in regard to their nature. Councilman and Tyzzer photo-

graphed these living cornea bodies with the ultraviolet light, and the structure came out as the chromatin structures of known living cells.

**Pathogenesis.—For Lower Animals.**—Various animals seem to contract the disease, or a modification of it, in nature. Horse-pox, sheep-pox, and cow-pox, all show similar pathological changes. Experimentally, probably all mammals are susceptible, though in varying degrees. Most of them are more sensitive to vaccinia than to variola. The epidermis of rabbits, for instance, shows a beautifully typical eruption after inoculation with vaccine virus, while material from smallpox eruptions produces only diffused redness. The corneal “take,” however, in both instances, is similar in intensity. Monkeys are equally susceptible to both forms of the disease.

**For Man.**—Without vaccination human beings seem to be equally susceptible to infection with variola, whatever their race or their condition in life or in whatever part of the world they live.

**Immunity.**—The immunity caused by successful vaccination is not permanent, and varies in its duration in different individuals. Although it usually gives protection for several years and may give it for ten or fifteen years, it is not well to count on immunity for more than one year, and whenever one is liable to exposure it is well to be vaccinated. If this vaccination were unnecessary it will not be successful, while if it is successful we have reason to believe the individual was open at least to a mild smallpox infection.

**Protective Substances Present in the Serum of Animals after Successful Vaccination.**—It has been frequently shown that the blood serum of a calf some days after an extensive vaccination possesses feeble protective properties, so that the injection of one or two liters of it into a susceptible calf would prevent a successful vaccination. A further and more convincing fact has been demonstrated by Huddleston and others, namely, that when active vaccine is mixed in certain proportions with serum from an animal which had just recovered from a successful vaccination, and the mixture is inoculated into a susceptible animal, there is no reaction.

**The Preparation of Vaccine.**—The following is the method employed at the New York City Health Department.

**Seed Virus.**—This may be prepared by one of the following methods:

1. *Glycerinized bovine virus* which has been ripened for two or three months. Such virus becomes attenuated after a varying number of calf-passages.

2. *Rabbit Virus.*—Rabbits vaccinated with stock bovine virus. This may also result in deterioration.

3. *Human Virus.*—This consists of the serum collected on sterilized bone slips from the vaccine vesicles of previously unvaccinated children. This produces good vaccine on calves; it is manifestly difficult to secure.

4. *Glycerinized vaccine* from calves which have been vaccinated with an emulsion of human vaccine crust. This also gives good virus, but it necessitates the constant collection of human crusts.

5. *Human-Calf-Rabbit Seed.*—This has been found to be the most economic, efficient, and reliable seed yet found by us. It is produced

as follows: Crusts are collected from healthy children about nineteen days after successful vaccination. These crusts are cut up and emulsified with boiled water to a mucilaginous paste. This humanized seed is inoculated into an area about 6 inches square upon the abdomen of a calf, the remainder of the calf being vaccinated in the ordinary way. The pulp from this special area is separately collected and glycerinized in the usual way. It is then tested bacteriologically and clinically. This bovine virus from human seed is now used in a dilution of 1 part to 12½ parts of normal salt solution to vaccinate rabbits. The seed is rubbed sufficiently upon the freshly shaven skin of the back. Five days after vaccination the pulp is removed with a curette, weighed and emulsified in a mortar with the following solution: glycerin 50 per cent., sterile water 49.5 per cent., and carbolic acid 0.5 per cent., in the proportion of 1 part of pulp to 8 parts of the solution. Four rabbits should yield from 15 to 20 c.c. of this emulsion, an amount sufficient to vaccinate one calf. The regular supply of vaccine is produced by vaccinating calves with this rabbit seed in the manner to be described.

**Animals.**—The preferable animals are female calves, from two to four months of age, in good condition and free from any skin disease. These can easily be vaccinated on the posterior abdomen and insides of the thighs by placing them on an appropriate table. It is possible that, on account of the character of the available supply, older animals may be desirable, but the calves take more typically and are more easily handled. When an animal is too old to be thrown and held without difficulty it may be vaccinated on the rump each side of the spine; but the skin there is tougher than on the posterior abdomen and insides of the thighs, and the resulting virus, though efficient, is not so easily emulsified.

**Vaccination.**—The hair should be clipped from the entire body when the animal is first brought into the stable and the calf should be cleaned thoroughly, including the feet and the tail. Just before vaccination the posterior abdomen and insides of the thighs are shaved and the skin beneath washed in succession with soap and water, sterilized water and alcohol, and then dried with a sterile towel. On this area there are now made superficial linear incisions with a sharp knife, about a fourth of an inch apart. After they have been made they should be dried with a sterile towel or with sterile cotton and rubbed with the charged slips. One or two slips, depending on the amount of virus each slip holds, should be sufficient for vaccinating each incision.

**Collection.**—On the fifth or sixth day, depending upon the rate of development of the vaccine vesicles, they should be ready for collection. The entire shaved area is washed with sterile water and sterile cotton, and the crusts are picked off. The soft, pulpy mass remaining is then curetted off with an ordinary steel curette and the pulp placed in a sterilized vessel. The pulp should be mixed with four times its weight of glycerin and water (50 per cent. glycerin, 49 per cent. water, 1 per cent. carbolic acid). This is done by placing the pulp in

a mortar and gradually adding the fluid. The more watery the pulp, especially if it is not to be used immediately, the smaller should be the proportion of glycerin. The emulsion so produced can then be put up for issue in vials. Capillary tubes require special means of filling, and small vials filled and corked answer the purpose admirably.

**Care of the Calves.**—The calves are inspected by a Department veterinarian at the time of purchase and during the period of detention previous to vaccination. After the vaccine has been collected the calves are immediately killed and their organs examined by the veterinarian. If, at autopsy, an animal be found tuberculous or otherwise diseased, the vaccine is discarded.

The vaccine stable at Otisville, N. Y., is a new building with screened windows and concrete floors and stalls, which are kept flushed with water to wash away the dejecta of the animals.

The calves stand upon raised racks of galvanized iron. They are fed on milk, no hay or straw being used for any purpose.

The calves are vaccinated and the vaccine pulp collected under careful aseptic precautions in a separate operating room, which has tiled walls and a concrete floor.

The vaccine pulp when collected is placed in sterile glass containers, sealed, packed in ice and shipped at once by express to the vaccine laboratory.

**Preparation and Testing of Finished Product.**—The mixture of vaccine pulp and diluent is pressed through a 40-mesh sieve with the pestle several times until there is no residue on the sieve. Then it is passed about twice through a 100-mesh sieve.

**Laboratory Tests for Purity.**—The following tests for purity are now made:

1. Plating upon agar and counting colonies of organism. This is done weekly for five weeks. After one to three weeks the plates usually show no growth, the carbolic acid and glycerin having killed off the extraneous organisms.

2. Inoculation of glucose broth in fermentation tubes for evidence of gas-forming organisms.

3. *Tests for Tetanus.*—Two tests are carried out at the same time, using two different anaërobic methods. After the cultures have incubated six days, they are filtered and guinea-pigs are inoculated with the filtrate, and are watched for ten days for evidences of tetanus.

4. *Test for Streptococcus.*—A guinea-pig is inoculated subcutaneously with the freshly prepared vaccine and watched for ten days for evidences of streptococcus infection.

**Clinical Tests for Potency.**—After all the laboratory tests for purity have been made and found satisfactory, *and not until then*, the vaccine is ready for use upon human beings, provided it be found to be efficient. To determine this point, fifteen inoculations of the vaccine are made upon previously unvaccinated children. These must all show a perfect take in order to pass the vaccine as up to standard. A clinical test of such vaccine is made every two weeks thereafter so long as the

vaccine is on the market. If one of these tests fails before the end of the period of guarantee, the vaccine is called in.

**Keeping of Vaccine.**—Bulk vaccine is kept in cold storage at a temperature of 4° to 8° F. below zero.

Vaccine which has been put up in packages ready for issue is kept in an ice-box at a temperature of 33° to 40° F.

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## CHAPTER XLV.

### RABIES. YELLOW FEVER.

#### RABIES.

**Introduction.**—Rabies (synonyms: Hydrophobia, Lyssa, Hundswuth, Rage) is an acute infectious disease of mammals, caused by a specific virus, and communicated to susceptible animals by the saliva of an infected animal coming in contact with a broken surface, usually through a bite. The name rabies (Latin) is given to the disease because of its most frequent and characteristic symptom—furor or madness. Hydrophobia (Greek, fear of water) is another name commonly used, which is also given because of a frequent symptom of the disease, the apparent fear of water. Lyssa is a Greek word meaning hydrophobia. Within the gray nervous tissue of rabid animals are peculiar protozoön-like structures known as “Negri bodies” which are diagnostic of rabies. The nature of these bodies is still a question of dispute (see below).

**Historical Note.**—Rabies is probably one of the oldest diseases in existence, but because of the occurrence of so few human cases, and because the disease develops so long after the bite, its source was for a long time not known nor was it recognized as a separate disease. Hippocrates does not mention it in his writing, but Aristotle about fifty years later (about 300 B. C.) speaks of its being purely an animal disease and being carried by the bite of one animal to another. Celsus, in the first century, was the first to give in writing a detailed description of human rabies. He speaks of it being produced by the bite of rabid animals and states that the wound must be thoroughly bathed and then burned with a hot iron in order to prevent the development of the disease, for after symptoms appear death always follows. As Celsus was not a physician he probably obtained his knowledge from writings which have since been lost. Other writers soon after gave very true descriptions of the symptoms and handling of the disease.

Many hundred years passed after this without adding anything to our knowledge of the disease, though authors on the subject were numerous. Van Sweiten in 1770 observed the paralytic form of rabies in human beings. At this time several authors, among them Morgagni and Zwinger, believed that the bite of a dog which was not suffering from rabies might produce the disease in man. In 1802 Bosquillon brought forth the original idea that belief in the existence of infectious material in rabies was a chimera and that hydrophobia was simply due to fright. This false idea had adherents for a long time; even now, by a few people, it is thought to be a true one.

Among the host of good observers who studied the disease during the latter part of the nineteenth century, Pasteur stands out as the discoverer, in 1880, of the fact that the disease may be prevented by inoculating gradually increasing doses of the virus into the person or animal bitten. This treatment with some modifications, the details of which will be given later, is still used, though many efforts have been made to develop an efficient serum treatment. Pasteur, as well as numerous other investigators, tried to discover the specific cause of rabies, but all of the results were negative. The importance of making a quick

diagnosis had become so evident that the efforts of many workers were directed toward this end alone.

Pasteur and his immediate followers relied for their diagnosis entirely upon rabbit inoculations, and this meant a fifteen to twenty days' wait before the patient knew whether or not the treatment he was receiving was necessary. In 1898 this time was shortened to about nine days in our laboratory by Wilson, who found that guinea-pigs came down with the disease much more quickly than rabbits. From time to time it has been thought that certain histological findings were diagnostic; for instance, the "rabid tubercles" of Babes, and the areas of "round- and oval-celled accumulation in the cerebrospinal and sympathetic ganglia" of Van Gehuchten and Nelis were said to be specific, but further study has shown that they are not absolutely specific for rabies. In many cases the whole picture of the grosser histological changes is sufficiently characteristic to warrant the diagnosis of rabies, but often it is not so.

It was not until Negri, in 1903, described certain bodies (Negri bodies) seen by him in large nerve cells in sections of the central nervous system, that anything was found which seemed absolutely specific for hydrophobia. Negri claims that these bodies are not only specific for rabies, but that they are probably animal parasites and the cause of the disease. We independently found the same bodies.

This work, especially so far as the diagnostic value of these bodies is concerned, has been corroborated by investigators in almost all parts of the scientific world, among them workers in our own laboratory who not only determined their worth in diagnosis, but investigated their nature.

In our work emphasis was placed upon the fact that the demonstration of the "Negri bodies" by our "smear method" (see p. 562) wonderfully simplified the process of diagnosis. As a result of our studies we concluded that the Negri bodies are not only specific for rabies, but that they are living organisms, belonging to the protozoa, and are the cause of the disease; giving as our reasons the following facts: (a) They have a definite characteristic morphology; (b) this morphology is constantly cyclic, that is, a definite series of forms indicating growth and multiplication can be demonstrated; (c) structure and staining qualities, as shown especially by the smear method of examination, resemble those of certain protozoa.

Since this report was published many more cases of rabies have been more or less studied by us and our former conclusions have been more firmly established. Indeed, the evidence as to animal nature of these cell inclusions seemed so convincing that Williams, in 1906, gave them the name *Neuroryctes hydrophobiae*.<sup>1</sup> Calkins has since studied these bodies and agrees with Williams as to their nature. He called attention to the similarity between their structure and that of the rhizopoda.

A number of observers, however, still believe that the Negri body as a whole is principally the result of cell degeneration and that the specific organism may be contained within it. Prowazek includes rabies with his "chlamydozoan diseases" (see p. 555). To anyone who has made a long and minute study of the two diseases, however, there can be no question in regard to the essential difference between the "trachoma bodies" and the "Negri bodies."

**Material and Methods for Study.**—In New York one may still frequently obtain fresh brains of rabid animals, from veterinary hospitals or from the laboratories handling this material. Two methods have been used in helping to study the principal site of infection.

- (1) Animal inoculations. (2) Sections and smears.

<sup>1</sup> Proceedings of the New York Pathological Society, 1906, vi, 77.



The first method is used as a decisive test in diagnosis when results from the second method are doubtful.

The technic of the *smear method* used at present in the Research Laboratory of the New York City Health Department is as follows:

1. Glass slides and cover-glasses are washed thoroughly with soap and water, then heated in the flame to get rid of oily substances.

2. A small bit of the gray substance of brain chosen for examination is cut out with a small sharp pair of scissors and placed about 1 inch from the end of the slide, so as to leave enough room for a label. The cut in the brain should be made at right angles to its surface and a thin slice taken, avoiding the white matter as much as possible.

3. A cover-slip placed over the piece of tissue is pressed upon it until it is spread out in a moderately thin layer; then the cover-slip is moved slowly and evenly over the slide to the end opposite the label. Only slight pressure should be used in making the smear, but slightly more should be exerted on the cover-glass toward the label side of the slide, thus allowing more of the nerve tissue to be carried farther down the smear and producing more well-spread nerve cells. If any thick places are left at the edge of the smear, one or two of them may be spread out toward the side of the slide with the edge of the cover-glass.

4. For diagnosis work such a smear should be made from at least three different parts of gray matter of the central nervous system: (1) From the cortex in the region of the fissure of Rolando or in the region corresponding to it in lower animals (in the dog, the convolution around the crucial sulcus); (2) from Ammon's horn, and (3) from the gray matter of the cerebellum.

5. The smears are partially dried in air and the method of fixation and staining given on p. 84 is applied to them.

With this method the Negri bodies stain magenta, their contained granules blue, the nerve cells blue, and the red blood cells yellow (Plate IX, Fig. 1).

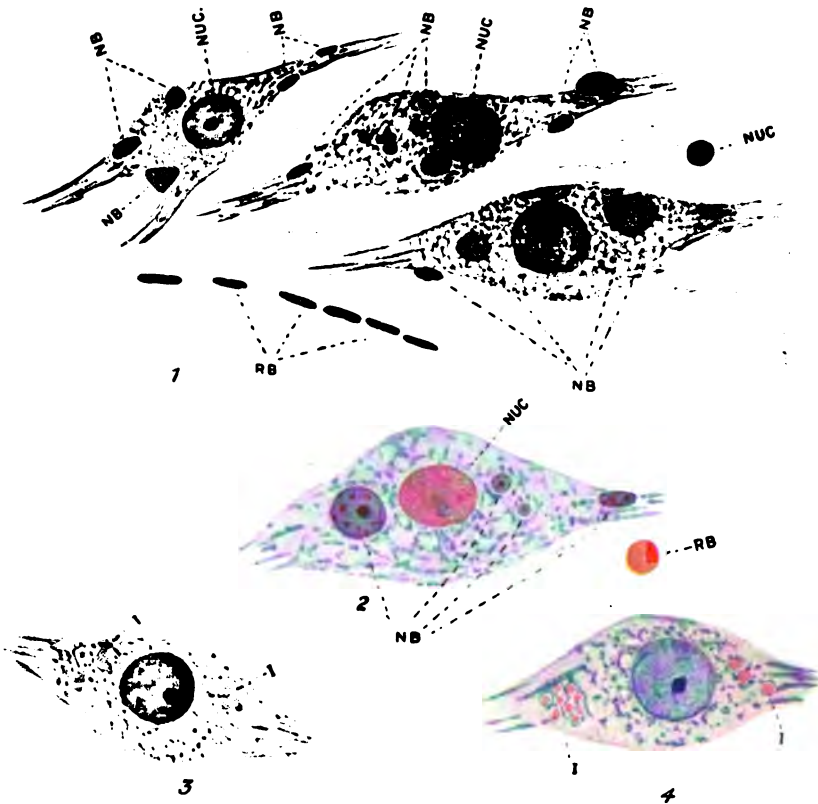
Other methods we have found useful for staining smears are: (1) Giemsa's (p. 82), by which generally the "bodies" are a blue and the contained granules are azur. The cytoplasm of the nerve cells stains blue also, but with a successfully made smear the cytoplasm is so spread out that the outline and structure of most of the "bodies" are seen distinctly within it. The nuclei of the nerve cells are stained red with the azur, the nucleoli a dull blue, the red blood cells a pink yellow, more pink if the decolorization is used (Plate IX, Fig. 2). The "bodies" have an appearance of depth, due to their refractive qualities. (2) eosin-methylene-blue method of Mallory (p. 84). With this method of staining, the cytoplasm of the Negri bodies is a magenta, light in the small bodies, darker in the larger; the central bodies and chromatoid granules are a very dark blue, the nerve-cell cytoplasm a light blue, the nucleus a darker blue, and the blood cells a brilliant eosin pink. With more decolorization in the alcohol the "bodies" are not such a deep magenta, and the difference in color between them and the red blood cells is not so marked.

In the technic of the *section* work (p. 84) the most important point is the time the material is allowed to remain in Zenker. According to our experience two hours' fixation is not enough, three to eight hours is very good, and with every hour after eight hours the results become less satisfactory. Left in Zenker overnight the tissue is granular and takes the eosin stain more or less deeply, both of which results interfere with the appearance of the tiniest "bodies," especially of the very delicate, minute forms found by us in sections from fixed virus.

The sections may be stained by the eosin-methylene-blue method of Mallory (p. 84). In the sections made in this way we have been able to demonstrate clearly very minute forms, as well as good structures in the larger forms. Giemsa's method for sections may also be used. Mann's method, recommended by others, has not given us such good results.

Harris has published a staining method for both sections and smears, which brings the larger bodies out clearly, but which does not seem to give enough differentiation between the smaller bodies and the nucleoli of the nerve cells.

## PLATE IX



A. W. WILLIAMS, DEL.

### Nerve Cells in Spreads from Ammon's Horn. Magnification 1200 diameters.

Figs. 1 and 2 from dog "street rabies" show "Negri bodies" (NB); Fig. 3 from non-rabic cat, and Fig. 4 from dog distemper show indefinite inclusion (I) that might be mistaken for Negri bodies by the inexperienced. Negri bodies are structured, more intensely staining, and more refractive. Figs. 1, 3, and 4 are stained by fuchsin-methylene blue mixture, which stains Negri bodies (NB) red with blue granules, nucleus (NUC) of nerve cells blue, and red-blood cells yellow (RB). Fig. 2 is stained by Giemsa's mixture, which stains Negri bodies a robin's egg blue with red granules, nucleus of nerve cells red, and red-blood cells salmon pink.



**Morphology of the Negri Bodies.**—The largest forms measured are about  $18\mu$  and the smallest about  $0.5\mu$ . They are round, oval, oblong, triangular, or ameboid. The latter are more numerous in the fixed virus of rabbits and guinea-pigs. Their structure is shown especially well in smears. Whatever the variety or species of animal infected, the bodies present the same general characteristic structure; *i. e.*, a hyaline-like cytoplasm with an entire margin, containing one or more chromatin bodies having a more or less complicated and regular arrangement.

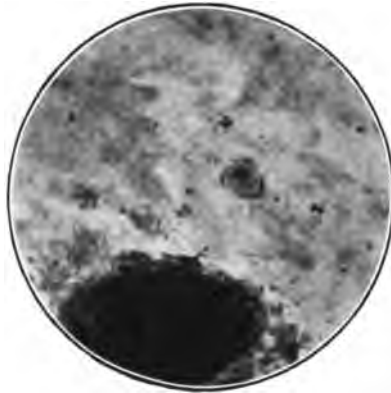


FIG. 196.—Negri body showing central chromatin with ring of small granules.  $\times 2000$ .

Their structure varies to a certain extent with their size. In fixed virus, with an occasional exception, only tiny forms are found. These are rounded or sometimes wavy in outline, as if possessing slight ameboid motion, sometimes elongated, extending along the rim of the host-cell nucleus, or along one of the nerve fibrils, as if moving there; with eosin and methylene blue they take a delicate light magenta stain, very similar to that taken by the small serum globules in the bloodvessels. Many of the organisms, however, show a small chromatin granule, situated more or less eccentrically, sometimes on the very rim of the body. In the larger forms the granule is large, in the smaller it cannot always be seen; some of the larger forms show from two to several granules and occasionally there is a body with the definite central body and the small granules about it.

*Detailed Characteristics of Structure in the Large Forms (Fig. 196).*—In smears, as well as in sections, the *cytoplasm* appears quite homogeneous; there is no evidence of a reticulum or of a granular structure outside of the definite chromatoid granules. The smears, however, have brought out one important point in regard to the cytoplasm more clearly than the sections, and that is that it is more basophilic than acidophilic in staining qualities. With the Giemsa stain, as we have already seen, it takes the methylene-blue stain more than the eosin red, and even with the simple eosin-methylene-blue stain the protoplasm appears as a deep magenta unless much decolorized.

In studying the *central bodies* of these organisms, as they appear in the smears, one of the first things noticeable is that they are not surrounded by a clear space—that there is no sign of a vacuolar appearance in the body as there usually

is in the sections. We notice next that in the great majority of the organisms the central body stands out clearly, as decidedly different in structure, and slightly so in staining qualities, from the chromatoid granules which surround it. The general type of the structure of the central body is that of many well-known protozoan nuclei; that is, the chromatin is arranged in a more or less granule ring around the periphery of the central body or nucleus, leaving an achromatic or more acid-staining centre in which is situated, generally eccentrically a varying-sized karyosome. There are a number of variations from this principal type, according to stage of development (Plate IX, Figs. 1 and 2).

Fragmented particles seem to be leaving the nucleus in certain forms, and in this way presumably the chromatoid granules are produced, thus forming chromidia.

The *chromatoid granules* are most frequently arranged in a more or less complete circle about the nucleus. They are somewhat irregular in outline and size, being occasionally ring-shaped, sometimes elongated, often in twos, due probably to active changes of growth and division. They take generally a more mixed chromatin stain than the chromatin of the nucleus.

**Evidences of Division.**—All stages in transverse division are seen. Many evidences of budding are also seen. The chromatoid granules seem to divide and pass out with part of the cytoplasm as a bud. This budding or unequal division appears to take place very early in the growth of the organism and to continue throughout growth until the parent body forms a mass of small organisms which may then break apart at the same time. The budding accounts for the number of small and large forms in a single cell (Plate IX, Fig. 1).

**Number.**—They vary in number according to the stage of the disease and to the infectivity of the part.

**Site.**—They are situated chiefly in the cytoplasm and along the fibers in the branches of the large nerve cells of the central nervous system. In parts of smears which are more broken up the bodies may appear as if lying free, and it is these bodies, if the pressure is not too great in smearing, that show the structure best. In some cases the bodies are distinctly localized in small scattered areas of the central nervous system. We have always found bodies in the spinal cord in abundance, but here they are especially prone to be localized in discrete groups of cells.

Manouëlian has found them in the ganglion cells of the salivary gland.

That the organisms are present in various glands of the body (salivary thyroid, suprarenal capsule, etc.) is shown by the virulence of emulsions from these organs. Cows' milk (Westbrook, McDaniel) and blood (Marie) have also been shown to be occasionally virulent.

**Cultivation of the Rabies Organism.**—Many attempts have been made to produce artificial cultures, but no reports have been so far corroborated. Noguchi, the latest claimant, states that he has obtained virulent cultures on the twenty-first transplant. Williams, Gurley, Krauss, Barbara, and Volpino have not been able to get the same results.

**Diagnosis of Rabies.**—In our laboratory, for the past thirteen years, or since we have used the smear method in routine diagnosis, there have been many thousand cases in all examined, including suspected rabies and controls.

In all of our work controlled by careful animal inoculations we have never yet failed to have typical rabies develop in animals inoculated with material showing definitely structured Negri bodies. Negative results after inoculation with such material must be interpreted at present as due to some error in technic, such as regurgitation, or hemorrhage at the time of inoculation, emulsion improperly made, not enough of the virulent material taken because of localization of the organisms, etc.

Possibly individual resistance of the animal inoculated might play a part. We have used principally guinea-pigs, and some of them have shown enough irregularity in regard to the time in which they have come down with the disease to suggest a varied individual susceptibility, if other factors can be ruled out.

On the other hand, material in which we have failed to demonstrate typically structured bodies has produced rabies. All of this material, however, since we have improved our technic, has shown suspicious small forms similar to those found in rabbit-fixed virus. But any decomposing brain may also show in smears, bodies very similar to these tiny forms, therefore it is difficult to rule out rabies in such cases. Of course the animal test will probably always have to be used with brains that are too decomposed to show any formed elements except bacteria, unless a reliable chemical test can be discovered. Brains from animals dying of distemper may show small non-structured forms, somewhat like "fixed virus" forms (Plate IX, Figs. 3 and 4).

So far we have not had rabies produced by fresh brains showing no Negri bodies and no suspicious forms, but a few observers have claimed that such material has produced the disease. Therefore, until we can standardize our technic, we must in all such cases use animal inoculations. We may, however, be reasonably certain that a case showing such negative material was not a case of rabies. We may summarize our knowledge in regard to the worth of the smear method in diagnosis as follows:

1. Negri bodies demonstrated, diagnosis rabies.
2. Negri bodies and suspicious bodies not demonstrated in fresh brains, not rabies.
3. Negri bodies not demonstrated in decomposing brains, uncertain.
4. Suspicious bodies in fresh brains, probably rabies.

The *localization* of the Negri bodies is an important point in making diagnoses. We have found well-developed bodies distinctly localized in different parts of the brain in several instances. In horses there may be small, widely scattered areas of well-structured forms throughout the cerebellum, while tiny, indefinite forms are scattered through the rest of the brain examined. In human brains well-developed forms are found in the corpus striatum and not in the rest of the brain. In several dogs the localization has also been marked.

**The Complement-binding Test in Rabies.**—This test has been tried by Heller (1907), Friedberger (1907), and Baroni (1908), with negative

results. Berry (1910), and Olmstead and Wilson, in 1916, in our Research Laboratory, went over this work thoroughly and obtained similar negative results.

**Effect of Chemical and Physical Agents on Rabic Virus.**—Rabic virus appears to become attenuated under certain conditions of temperature; indeed, if it be subjected for about an hour to 50° C. or for half an hour to 60° C., its activity is completely destroyed. A 5 per cent. solution of carbolic acid, acting for the same period, exerts a similar effect, as do likewise 1 to 1000 solutions of bichloride of mercury, acetic acid, or potassium permanganate. Cumming has shown that formalin is especially deleterious to the virus. The virus also rapidly loses its strength by exposure to air, especially in sunlight; when, however, protected from heat, light, and air it retains its virulence for a long period.

The virus is readily filtered through all grades of Berkefeld filters, and from the glands through the coarser Chamberland. Poor and Steinhardt have shown that the filtered gland and brain virus seem to have similar characteristics.

**Pathogenesis.—Natural Infection.**—The disease occurs in nature among the following animals given in order of their frequency: dogs, cats, wolves, horses, cows, pigs, skunks, deer, and man; in fact, as all warm-blooded animals are more or less susceptible to inoculations, all may presumably contract the disease when an open wound is brought in contact with infectious material of a rabid animal.

Rabies occurs in almost all parts of the world. It is most common in Russia, France, Belgium, and Italy; it is not infrequent in Austria and in those parts of Germany bordering on Russia. In this hemisphere it is infrequent in Canada, but in the United States the cases are increasing in numbers. In Mexico and South America it occurs occasionally; while in England, North Germany, Switzerland, Holland and Denmark, because of the enforced quarantine laws, and of the wise provision that all dogs shall be muzzled, it is extremely rare. In Australia it is unknown, probably because the law that every dog imported into the island must first undergo a six months' quarantine has always been enforced. Since 1915 it has decreased markedly in New York City due to the fact that the muzzling ordinance has been enforced.

In this connection the question as to how long the sputum of a rabid dog may remain virulent after it drops from the animal is an interesting one. A case came under our observation in 1906 which illustrates this point. A child of six years came down with typical rabies in a neighborhood where there had recently been several cases of canine rabies, but no history of a bite could be obtained. The parents were sure she had not been bitten. Six weeks before, however, the child had fallen in the street and cut her cheek severely on a jagged stone. The wound was cauterized and healed without further trouble. A mad dog had been on that street just before this occurred. It is reasonable to suppose that the stone had on it some of the sputum from that dog, and so the child was infected. Such a case would not occur very often, but the possibility should be considered.

In regard to the question as to whether the bite of apparently healthy animals may give the disease, it may be said that, judging from laboratory experiments,

some animals may have a light attack of the disease and recover spontaneously; though such cases, if they occur, are probably extremely rare. That the bite of an infected animal may give the disease before that animal shows symptoms has been proved. Fifteen days is the longest time reported between a bite and the appearance of symptoms in the dog. Therefore, if an animal is kept under observation three weeks after biting another, without developing symptoms, he may be pronounced free from suspicion.

Neither age, sex, nor occupation has any specific effect. The time of the year seems to have little effect, though most cases are said to occur during the summer months. The numbers vary with different years.

The certainty with which the disease may be produced after a bite and the rapidity of its development have been found to be governed by three factors: (1) the quantity of the rabic virus introduced; (2) the point of inoculation; (3) the strength of the virus as determined by the kind of animal which affords the cultivation ground for the growth of the organism. It is a matter of common observation that in man slight wounds of the skin of the limbs and of the back or wherever the skin is thick and the nerves few either produce no results, especially when bites are made through clothes, or are followed by the disease after an extremely long period of incubation; while in lacerated wounds of the tip of the fingers where small nerves are numerous or where the muscles and nerve trunks are reached, or in lacerated wounds of the face where there is also an abundance of nerves the period of incubation is usually much shorter and the disease generally more virulent.

These facts explain why only about 16 per cent. of human beings bitten by rabid animals and untreated appear to contract hydrophobia.

Since the establishment of the Pasteur treatment for the disease, the percentage of developed cases after bites is very much less—a fraction of 1 per cent.

**Symptoms.**—There is always a decided incubation period after the bite which varies within quite wide limits, but in the majority of cases it is from twenty to sixty days. Any period after six months is an exception; the shortest we have on record is fourteen days and the longest authentic period is seven months. A very few apparently authentic cases have been reported as developing in about one year, but reports of any time beyond this must be received with doubt.

The symptoms may be divided into three stages: (1) The prodromal or melancholic stage; (2) the excited or convulsive stage; and (3) the paralytic stage.

When the second stage is the most pronounced the disease is called furious or convulsive rabies; when this stage is very short or practically lacking and paralysis begins early, the disease is called dumb or paralytic rabies.

In the dog the principal symptoms of each form may be summarized as follows: (a) *Furious rabies*: change of behavior, biting (especially at those to whom the animal has been affectionate before), increased aggressiveness, characteristic restlessness, loss of appetite for ordinary food, with desire to eat unusual things, intermittent disturbance of consciousness, paroxysms of fury, peculiar howling bark, rapid emaciation, paralysis, beginning in the hindlimbs, death in great majority of cases in three to six days (exceptionally slightly longer) after the beginning of symptoms. (b) *Paralytic rabies*: short period



of excitation, paralysis of the lower jaw, hoarse bark, appetite and consciousness disturbed, weakness, with paralysis spreading in great majority of cases, and death four or five days after first symptoms. There may be a number of cases showing transition types between these two forms.

**In Human Beings.**—*Furious Rabies.*—The first definite symptoms are difficult and gasping breath with a feeling of oppression and difficulty in swallowing, the latter the most characteristic symptom. It is caused by convulsive contraction of the throat muscles. The attacks are brought out when attempting to drink or swallow. The very thought of drinking may bring one on; and though there is no fear of water itself, there is fear of taking it because of the effect it produces. The convulsive attacks finally become more or less general over the whole body; in certain cases some parts are more affected by reflex excitation than others; for instance, there may be slight or no photophobia, while in exceptional cases, more frequently in dogs, the hydrophobia is also absent.

Most of the special reflexes are increased. Pupils become irregularly contracted and widened until they finally remain fixed.

Human beings are seldom dangerous to the people about them. In their convulsions they may bite things placed between their teeth, but not otherwise. At this time there is an increased flow of saliva, and one should avoid the contact with this in opened wounds. It may be so increased that the patient may try to get rid of it by taking it from the mouth with the hand and throwing it about. As a general thing, however, the patient has full possession of his senses between the convulsive attacks until very late in the disease.

The temperature is increased from 38° to 40° C., at first with morning remissions. Just before death it may rise as high as 42.8° C. (In lower animals the temperature sinks below normal just before death.) The pulse is generally over 100 and is irregular. This stage lasts from one to four days. Death may occur during a convulsion, but more often there is a *paralytic stage*, which lasts from two to eighteen hours. The convulsions become less frequent and the patient becomes weaker until finally there is a complete paralysis. At the beginning of this stage the patient may be able to drink water better than formerly. Death may occur at any time through paralysis of the heart or respiratory centre.

*Paralytic Rabies.*—This form occurs quite seldom in human beings, more frequently in dogs, but not so often as the convulsive form. It is supposed to occur in humans and dogs after a more severe infection. Instead of periods of convulsions, the various muscles simply tremble and become gradually weaker until complete general paralysis supervenes. Sometimes paralysis develops very quickly and may be general before death from syncope or asphyxia occurs. This form generally lasts longer than ordinary rabies. Between these two typical forms of rabies there are many different types, giving quite varied pictures of the disease.

**Length of the Disease.**—The majority of the cases of furious rabies die on the third or fourth day after the symptoms show themselves. The limits of the reported cases are one to fifteen days, though there are reports of only one or two cases dying on any day after the ninth to the fifteenth. As the time when the symptoms really begin is difficult to notice, these statistics are probably only approximately correct. In paralytic rabies the average time in which death occurs is five days.

**Treatment.**—The old treatment of rabies consisted simply in encouraging bleeding from the wound, or in first excising the wound and then encouraging bleeding by means of ligatures, warm bathing, cupping-glasses, etc.; the raw surface was then freely cauterized with caustic potash, nitric acid, or the actual cautery. It is doubtful whether the disease ever manifested itself after such heroic treatment if the wounds

were small and the treatment was begun soon after the bite; but when the wounds were numerous or extensive the mortality was still high. As it was often impossible to apply cauterization to the wound rapidly or deeply enough to insure complete destruction of the virus, Pasteur and others were led to study the disease experimentally in animals with the hope of finding some means of immunization or even cure. These investigations finally resulted in the discovery of methods of preventive inoculation applicable to man.

**Pasteur's Method of Preventive Inoculation.**—Pasteur's treatment is based upon the fact that rabic virus may be attenuated or intensified under certain conditions. He first observed that the tissues and fluids taken from rabid animals varied considerably in their virulence. Then he showed that the virus may be intensified by successive passage through certain animals (rabbits, guinea-pigs, cats) and weakened in passing through others (monkeys). If successive inoculations be made into rabbits with virus, either from the dog or the monkey, the virulence may be so exalted beyond that of the virus taken from a street dog, in which the incubation period is from twelve to fourteen days, that at the end of the fiftieth passage the incubation period may be reduced to about six or seven days when it remains fixed. This "*fixed virus*" was used by Pasteur and those after him in his preventive treatment because the dose could be more definitely regulated by subsequent attenuation or dilution.

**Original Method.**—A series of spinal cords from rabbits dead from "fixed virus" infection are cut into segments and suspended in sterile glass flasks plugged with cotton stoppers and containing a quantity of some hygroscopic material, such as caustic potash; these are kept at a temperature of about 22° C. The cord when taken out at the end of the first twenty-four hours is found to be almost as active as the fresh untreated cord; that removed at the end of forty-eight hours is slightly less active than that removed twenty-four hours previously; and the diminution in virulence, though gradual, progresses regularly and surely until, at the end of the eighth day the virus is inactive. Pasteur began his treatment with an emulsion of the cord kept until the fourteenth day. A certain quantity of this was injected into the animal that had been bitten; this was followed by an injection of an emulsion of a twelve-day cord; and so on until the animal had been injected with a perfectly fresh and therefore extremely active cord, corresponding to the fixed virus. Animals treated in this way were found by Pasteur to be absolutely protected, even against subdural inoculation with considerable quantities of the most virulent virus, and thus Pasteur's protective inoculation against rabies became an accomplished fact. As it would be undesirable to inject any but persons who had actually been bitten by a rabid, or presumably rabid animal, Pasteur continued his experiments in order to see whether it would not be possible to cure a patient already bitten. He carried on, therefore, a series of experiments which led to the discovery that if the process of inoculation be begun within five days of the bite in animals in which the incubation period was at least fourteen days, almost every animal bitten can be saved; and that even if the treatment be commenced at a longer interval after the bite a certain proportion of recoveries can be obtained. Thus the application of this method of treatment to the human subject was not tried until it had been proved in animals that such protection could be obtained and that such protection would last for at least one year and probably longer.

The chance of success in the human subject appears to be even greater than

in the dog or rabbit. Man's period of incubation is comparatively prolonged. Thus there is an opportunity of obtaining immunity by beginning the process of vaccination soon after the bite has been inflicted, the protection being complete before the incubation period has passed.

**Present Administration of Pasteur's Treatment in Human Beings.**—The original method of Pasteur in its entirety was soon adopted in many lands, and his results were corroborated. Before long, however, a number of modifications were suggested by different observers, some slight, others more fundamental. Some have been widely used, such as Högyes' dilution method; others have had a limited application in lower animals and are probably only of theoretic interest as regards man. Such are the intravenous inoculation of brain emulsions from street rabies into herbivora (Nocard and Roux, Protopopoff), and the intraperitoneal inoculations of large doses of fully virulent fixed virus into dogs, cats, or rabbits (Hellman, Heim, Remlinger). Immunity has been produced also in rats by allowing them to feed on rabid brains (Fermi, Repetto, Remlinger).

Högyes in Budapest was one of the first to use a different procedure. He claimed that the virus by Pasteur's method was attenuated only through the death of some of the specific organisms, that is, that there were simply fewer living organisms in the early doses given than in the later, and that therefore the same result might be obtained perhaps with even more accurate dosage by giving gradually decreasing dilutions of a fresh virulent cord. By diluting sufficiently he obtained a mixture which when inoculated did not produce rabies in the test animals, a result similar to that following an 8- to 10-day dried cord. This dilution he used for the first inoculation and gradually stronger dilutions for the succeeding ones.

Other methods of attenuating or diluting fixed virus have been used, such as exposure to the action of heat, cold, gastric juice, glycerin, or carbolic acid.

The mixed treatment with specific serum and vaccine has also been employed, chiefly by Marie, by Remlinger, and by Babes.

**Methods of Attenuation by Gradual Drying.**—Pasteur's classic method has undergone modifications in three general directions: (1) lengthening or shortening the period of treatment; (2) starting the inoculations with a less attenuated cord; (3) increasing or decreasing the amount given at each injection. The method of drying the cords, however, has remained essentially the same as that used by Pasteur.

The cord is removed by a modification of the method of Oshida in the following manner: Strict asepsis is preserved. The rabbit when completely paralyzed (seventh day) is killed by gas or chloroform and is dropped into a 5 per cent. solution of carbolic acid for 5 minutes. It is then removed, the excess of carbolic solution is drained off, and an incision through the skin at the upper and inner part of the thigh is made. The skin is loosened by cutting around the lower portion of the trunk. It is then pulled by the hands toward the upper extremity of the animal and over the head to the ears, leaving the back exposed

and sterile throughout the entire length of the spine. The spine is then divided transversely near each extremity by bone-cutting forceps. The muscles are cut through about these areas so the spine may be more easily reached. With a long wire probe swabbed with cotton at one end the cord is pushed upward from its canal, freed from its nerves and membranes. The spine is steadied by lion-jawed forceps. The cord curls in a spiral as it emerges and rests on the sterile muscles of the neck. It is lifted with forceps, placed in a Petri dish and cut in two. A small piece is cut from one end and is dropped into a tube of broth to test its purity. A ligature with one long end is placed about each piece, both of which are then hung in a drying bottle (Fig. 197).



FIG. 197.—One corner of constant temperature room showing drying bottle containing fixed virus cords being prepared for vaccine.

**DRYING THE CORD.**—The drying bottles are sterile aspiration bottles with both openings plugged with cotton. A layer 1 inch high of sticks of caustic potash covers the bottom, and the pieces of cord are suspended from the top cotton plug by their attached ligatures. The bottles are then labeled and placed in the constant temperature room (Fig. 197) or incubator, which is kept at a temperature of about  $22^{\circ}$  C. ( $70^{\circ}$  F.). After twenty-four hours' drying the cord is known as one-day cord; after two days, two-day cord, etc. Pieces of cord cut off at any time and put into glycerin will retain about the same strength for several weeks. This procedure is followed in regions where there are few cases of rabies, and the daily killing of rabbits to keep up the vaccine would be a large expense. It may also be followed where treatment is sent by mail.

The New York City Health Department used Pasteur's first schemata, with modifications, up to January, 1906, when they began treatment with a ten- and nine-day cord and finished with a one-day. They continued with this until August, 1913. Since then they have been using the

more intensive method of the Hygienic Laboratory at Washington. From 1906 to 1913 inclusive they treated 4282 cases infected by rabid animals, with a total mortality of 0.54 per cent. and a corrected mortality of 0.19 per cent. They have had 7 cases of definite paralysis with 2 deaths; 6850 cases in all, including those not bitten by rabid animals, were treated.

Since it had been found that fresh rabbit-fixed virus inoculated subcutaneously into man is apparently harmless, the Berlin Institute, with the hope of obtaining an earlier immunization and a shorter treatment, began to give still earlier cords. In 1901 it began with the eight-day cord on the first inoculation, and was inoculating a two-day cord on the eighth day of treatment. Its treatment lasted twenty-one days. This method was adopted at the Hygienic Laboratory in Washington in 1908, with slight variations for the different degrees of bites. Now only the intensive schema is used for all cases as follows:

Days.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Age of cord.		8	4	5	3	3	2	2	1	5	4	4	3	3	2	2	4	3	2	3	2	1
		7	3	4																		
		6																				
Amount injected in c.c.	Adults.	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
	5 to 10 years	2.5	2.5	2.5	2.5	2.5	2	2.5	1.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
	1 to 5 years	2.5	2	2.5	2	2	1.5	2	1	2.5	2.5	2.5	2	2	2	2	2.5	2.5	2	2	2.5	2

Each dose contains 1 cm. of the indicated cord.

The New York City Health Department has been using this schema since August, 1913, for severe head and face bites, but it reduces all doses by one-fifth, *i. e.*,  $\frac{1}{5}$  cm. of the indicated cord is emulsified in 3 c.c. of normal salt solution, and  $2\frac{1}{5}$  c.c. of this emulsion is inoculated. It also substitutes a two-day cord for the one-day cord of the eighth and twenty-first day in other than severe cases. In cases with very slight wounds which have begun treatment immediately the inoculations are carried only as far as the fifteenth day.

The inoculations are made subcutaneously usually over the abdomen.

**Treatment by Mail.**—The New York City Health Department was the first to send out treatment by mail to physicians for their own patients. Full directions are sent in the mailing case. One-fourth per cent. of carbolic acid is added to the emulsions prepared as above for the first three days' treatment; 20 per cent. glycerin is added to all other emulsions. The carbolic acid and the glycerin are added as preservatives, and are therefore omitted when the vaccine is administered to patients at the laboratory.

The results from the treatment sent in this way seem to be equally as good as those from the treatment administered at the laboratory.

**More Intensive Treatment.**—In Berlin, where intensive treatment has been longest used, they began to employ even fresher cords for beginning doses because they continued to have late deaths, though not quite

so often, after the more intensive methods they were using. Since 1910 the Pasteur Institute in Berlin has been using the following schema:

Days . . . . .	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Age of cords . . . . .	3	2	1	1	3	2	1	1	3	2	1	1	3	2	1	1	3	2	1	1	1

The dose is 2 c.c. of cord emulsion (1 part of cord in 5 parts of sterile physiological salt solution) inoculated once a day into the subcutaneous tissue of the abdomen. Children and adults receive the same dose.

Simon gives the following statistics of the results of Berlin's increasingly intensive methods:

BERLIN STATISTICS.

Period.	Age of cord used for beginning inoculation.	Cases.	Paralysis.	Mortality.	Per cent.
I. 1898-1906	Chiefly 8-day cord . . .	2896	0	21	0.7
II. 1906-1909	4-day cord. Sometimes 3-day cord . . . . .	1490	2	7	0.47
III. 1909-1910	3-day cord for all cases . .	819	3	5	0.6

Several others institutes are employing very intensive treatments, but their cases are still too few for consideration. Other directors still use the older methods.

**Rapid Drying of Rabies Virus.**—Recently Harris, of St. Louis, has published a new method of drying rabies virus and of regulating the dosage.

**TECHNIC.**—The brain and cord are removed aseptically and ground up in a sterile mortar with a sufficient quantity of CO<sub>2</sub> snow thoroughly to freeze the tissue. The frozen nerve tissue and snow are then placed in a Scheibler jar over H<sub>2</sub>SO<sub>4</sub>, the jar being kept in a Frigo apparatus. A vacuum of from 5 to 2 mm. is produced in the jar, which is then kept at the temperature of 18° C. by an ice and salt mixture for a sufficient length of time to dry thoroughly the nerve substance, which then appears as a dry powder. About two days are required for one brain and cord, which lose about one-half of their virulence in the process. The powder is then sealed in tubes *in vacuo* and kept at a temperature below 0° C. until required for use. It has been found that by keeping the powder thoroughly dry and cold practically no further loss of virulence occurs for at least six months. Before storing the virus for use its strength in units is computed, the unit being the minimal infecting dose (M. I. D.) for a rabbit when injected intracerebrally.

The advantages claimed for this method are: (1) the ease and economy with which a large amount of virus can be prepared, it being necessary to prepare the virus for use even in large laboratories only at intervals of several months; (2) the possibility of more accurate dosage for the patients; (3) a shortened period of treatment; and (4) the inoculation of more virus units. The required amount of powdered virus is weighed out each morning, and the necessary dilutions in salt solution for the various patients are made from this.

Up to October 13, 1913, Harris had treated 240 cases exposed to infection from dogs, in which the diagnosis of rabies was either proved by laboratory methods or strongly probable from veterinary diagnosis. Of this number one patient, who had started treatment six days after the bite, died of rabies during the period of observation. No cases of paralysis have been reported.

We must wait for further statistics before being able to judge of the efficiency of this method.

**FIXED VIRUS MODIFIED BY DIALYSIS.**—Cumming, of Ann Harbor, has devised a method of antirabic vaccination, by which he uses fixed virus which has been rendered avirulent by dialysis.

The emulsion of fixed virus is placed in collodion sacs (prepared by the Novy method and sterilized in the autoclave at 105° C. for twenty minutes) and dialyzed in distilled water for from twelve to twenty-four hours. The resulting vaccine does not produce rabies on intracranial inoculations, but does produce immunity on subcutaneous inoculations. Experiments by Cumming on rabbits show that whereas the original Pasteur method protects against only twice the minimum lethal dose (minute directions for obtaining the M. L. D. are given) injected intracerebrally, and the Högyes method against one and one-half times the fatal dose, the dialysis method protects against at least three times the fatal dose. He also claims that immunity is produced at an earlier date than by the other methods. Treatment (2 c.c. of the vaccine) is given daily for from fifteen to twenty-five days. Cumming reports over 800 cases (62 per cent. bitten by animals proved to have been rabid) treated without a death and without complications.

We must wait for further practical testing of this interesting method before deciding as to its comparative worth.

**Marie's Method.**—For several years past the use of virus serum mixture has been in vogue at the Pasteur Institute in Paris, the technic of which is as follows:

1 gm. of the medulla of a rabbit dead of fixed virus is finely emulsified with 9 c.c. of 0.8 per cent. salt solution and filtered through linen. Two c.c. of this emulsion and 4 c.c. of antirabic serum (obtained from sheep, and inactivated at 56° C. for thirty minutes) are carefully mixed after standing for a time. Six c.c. of this mixture, which contains an excess of virus is injected into the patient. These injections are repeated on the next three days, after which the treatment proceeds according to the regular Pasteur schema, beginning with the use of a six-day cord on the fifth day. The antirabic serum is obtained from sheep which have been subjected to a long and strong course of treatment with fixed virus. It is claimed that a quicker immunity is produced by the serum-virus mixture than by the original Pasteur scheme, an advantage of especial value in the treatment of cases liable to become infected with a short incubation, such as bites on the head.

**Antirabic Serum.**—The possibility that the serum of animals immunized against rabies contains protective substances was suggested by Pasteur as early as 1889. The following year Babes recommended the use of the serum of vaccinated animals in combination with the Pasteur treatment. Since then the study of the amount and character of the antibody content of animals immunized against rabies has been carried on more or less extensively both from the theoretic and the practical sides.

It was hoped that a serum could be obtained that would effect a cure for developed rabies just as diphtheria antitoxin does for developed diphtheria. But such a definite applicability of the serum has not developed. It was soon found that, while serum of certain vaccinated animals possessed the property of neutralizing rabies virus *in vitro*, it had only a slight inhibiting power when inoculated into the living animal, and apparently no action at all by any method of inoculation after the disease had become manifest. Babes still claims, however, that the serum has enough effect *in vivo* to be used in treatment, and his serum treatment is based upon this claim. He gives as his reason for employing serum at the end of treatment that he wishes to introduce into the patient at the time he most needs it the largest amount of antibodies. He also claims that the serum so given will prevent or cure the occasional paralyses which occur during treatment.

Those who did not agree with Babes were led to test the practical use of the serum combined with the beginning vaccine inoculations.

Remlinger, Marie, and others showed that a serum-virus mixture with a slight excess of virus will protect an animal against infection into the anterior chamber of the eye when inoculated during the three days following the vaccination. Thus Marie showed that immunity is produced more quickly by these unsaturated mixtures of virus and serum than by the virus alone. If a surplus of serum is present the animals are not protected from a later infection.

Marie, who has used the serum in his treatment of humans since 1904, prepares it as follows:

The brains of two rabbits dying from fixed virus infection are finely rubbed up with physiologic salt solution in the proportion of 20 gm. in 180 c.c. This emulsion is filtered through fine cloth and heated for one-half hour at 37° C. Sheep are used for the inoculation. Each sheep receives intravenously 30 c.c. (3 gm. fixed virus) a week for six to eight weeks. Thirteen days after the last inoculation the first blood is drawn. Then in a period of two weeks, at 4 bleedings, 200 c.c. of blood are drawn. After a fourteen-day pause another series of inoculations are given and the animal is ready for another series of bleedings. From each animal yearly about 3 liters of antirabic serum are obtained. A strong serum is one that neutralizes 40 virus units in 1 c.c.

A *virus unit* is 1 c.c. of five times the dilution of fixed virus that will surely kill a rabbit inoculated intracerebrally, *e. g.*, the unit of a fixed virus that will surely kill a rabbit in 1 to 500 dilution is 1 c.c. of a 1 to 100 dilution.

The nature of the antibodies in rabies serum has been the subject of many studies. Fermi and a few others claim that the antibodies are not specific. They say that they can obtain a similar serum after the inoculation of normal brain emulsions. Some even use normal brain emulsions in the treatment of their lighter cases.

Certain investigators (Kraus, Marie, and others), while not able to corroborate all of these claims, have found that the sera of certain animals which are more or less refractory to rabies possess a small amount of rabicidal strength; *e. g.*, 0.5 c.c. of normal chicken serum



mixed with one unit of fixed virus (1 c.c. of 1 to 100 dilution) causes the latter to become neutral in eighteen hours.

All species of animals tried produce the specific antibodies, but not to an equal degree. Human beings and monkeys are said to have more antibodies after vaccination than rabbits.

Centanni showed that immediately after vaccination the animal is not fully protected, though its serum may contain antirabic qualities, while later the animal is immune, though its serum may not be able to neutralize the rabies virus. These facts point to a cellular immunity.

**Results of Antirabic Treatment.**—On the whole the results of protective inoculations against rabies are marked. One has only to compare the statistics of mortality after bites from animals suffering from hydrophobia with those given after any of the methods of treatment employed to see the benefit. As regards the best method to use, the case is different. With many methods tried in many lands on a large number of cases, it would seem that we should be able by this time to determine their comparative worth. But the trouble is that the improvement on the whole is not great and the statistics are not kept uniformly or minutely enough to draw trustworthy comparisons.

A slight decrease in mortality has been shown in the statistics from most of the antirabic institutes of the world.

But these figures tell us little about the actual value of the different methods. In order to be able better to judge, the statistics should uniformly give many more details. Some institutes give such details, others do not. Until some such scheme as the following is carried out by all, we must change cautiously a treatment that has already given good results.

1. Diagnosis of biting animal:
  - (a) Rabies, (b) probably rabies, (c) questionable, (d) not rabies, (e) nothing known.
2. Manner of making diagnosis:
  - (a) By animal inoculation, (b) by microscopic examination, (c) by clinical diagnosis.
3. Site and character of bites (*e. g.*, number, depth, laceration, protected by clothing, etc.):
  - (a) Head, (b) hands, (c) other parts of body.
4. Time elapsing between bite and beginning of treatment.
5. Method of treatment used.
6. Complications during or after treatment, particularly paralysis.
7. Character and time of death.

That the time after the bite makes a great difference is shown by the following table:

Time intervening between bite and beginning treatment.		Number of cases treated.	Death.	Percentages.
Babes	1 to 2 days	3406	3	0.088
	3 to 5 days	2541	2	0.077
	5 to 6 days	809	1	0.124
Diatroptoff	1 week	4602	26	0.560
	2 weeks	961	16	1.660
	3 weeks	313	10	3.190

**IMMUNITY.**—The immunity in human beings produced by the anti-rabic treatment apparently lasts a variable time. That it may not last more than fourteen months is shown by the history of one of our cases. The patient was an assistant in a hospital for dogs. He was given eighteen days' treatment after a light wound in the hand from a rabid dog. Fourteen months later he came down with typical hydrophobia. Since his treatment he had become very careless with cases of rabies, exposing wounded hands to saliva because he considered himself immune. He was warned that there might be danger. Six weeks before his death he put a wounded hand into the mouth of a rabid animal. There is little doubt but that this is a case of reinfection after loss of protection from the treatment rather than one of delayed hydrophobia.

Marie has found complete immunity in dogs eighteen months after treatment.

**III Effects of Treatment.**—**LOCAL.**—There is only slight local discomfort, increased a little if the emulsion contains glycerin. During the second week an erythema often appears about the point of inoculation, which Stimson regards as a manifestation of hypersusceptibility to foreign nerve tissue. It disappears in a few days.

**CONSTITUTIONAL.**—Ever since the beginning of treatment occasional non-fatal affections of the nervous system have been reported, which occurred during or shortly after the course of treatment. These have varied in degree all the way from a slight neuritis, through paraplegias to paralyses of various parts of the body. Very occasionally the paralyses are marked and the patient dies. Cases of true paralytic rabies which may occur within the period required for the establishment of immunity by the treatment must be differentiated from cases occurring as a result of treatment.

Simon published an extensive report of 84 cases occurring during the years 1888 to 1911 inclusive, and Fielder gives a corroborative report in 1916. Simon classifies the cases collected by him according to the diagnosis of the biting animal, with the mortality in each group as follows:

Positive group.		Probable group.		Questionable group.		Negative group.		Not known group.	
Number cases.	Per cent.	Number cases.	Per cent.	Number cases.	Per cent.	Number cases.	Per cent.	Number cases.	Per cent.
25	29.76	11	13.0	21	25	17	20.23	10	11.9
(2)		(4)		(5)		(3)		(5)	

Nineteen deaths occurred, as seen from the figures in parentheses, or 22 per cent. of the 84 cases.

In analyzing the effect of different methods of treatment on paralyses, Simon gives the following summary:

	Number of cases treated.	Cases of paralysis.	Proportion.
Classic Pasteur method . . . . .	32,676	6	1 in 5446
Modified Pasteur method . . . . .	8,657	16	1 in 541
Högyes method . . . . .	51,417	3	1 in 17139

It is seen that the number of paralyses following the Högyes method are markedly less than those following the other methods.

From the studies so far made of these paralyses the possibility of there being different causes for different cases cannot yet be ruled out. The chief theories advanced as to factors in producing the condition are six:

1. Due to "laboratory rabies" from the fixed virus vaccine inoculated.
2. Due to "modified rabies" resulting from the treatment on the street virus infection.
3. Due to a toxin produced by the rabies organisms.
4. Due to infection with extraneous organisms introduced with the virus during treatment.
5. Due to psychological disorders.
6. Due to the inoculation of a foreign protein followed by an anaphylactic reaction.

**The Cauterization of Infected Wounds.**—We believe that in cases in which the Pasteur treatment cannot be applied great benefit may be derived from the correct use of cauterization with *fuming nitric acid*, even twenty-four hours after infection, and that even in cases in which the Pasteur treatment can be given, an *early* cauterization will be of great assistance as a routine practice and should be very valuable, as the Pasteur treatment is frequently delayed several days for obvious reasons, and then does not always protect. In the case of small wounds all the treatment probably indicated will be thorough cauterization with nitric acid within twelve hours from the time of infection. Our experience in dealing with those bitten by rabid animals goes to show that physicians do not appreciate the value of thorough cauterization of the infected wounds.

**Preventive Measures in Animals.**—Far more important than any treatment, curative or preventive, for hydrophobia in man is the prevention of rabies in dogs, through which this disease is usually conveyed. Were all dogs under legislative control and the compulsory wearing of muzzles rigidly enforced for two years where rabies prevails, hydrophobia would practically be stamped out. This fact has been amply demonstrated by the statistics of rabies in countries (*e. g.*, England) where such laws are now in force. New York City has recently had some measure of success in enforcing such laws.

### YELLOW FEVER.

Yellow fever is an acute infectious disease of tropical countries, with no characteristic lesions except jaundice and hemorrhage. Other lesions that exist are those common to toxemia. One attack usually produces complete immunity.

**Historical Note.**—There have been many extensive studies on the etiology of this disease with numerous announcements of the discovery of its specific cause. Not one of the latter, however, has been corroborated. The *Bacillus*

*icteroides* of Sanarelli (1897), found in the circulating blood and in the tissues of most yellow-fever patients, was thought by many to be the real organism, and for some time it was the subject of most minute studies with the result that it, too, has been placed with the rejected organisms.

The epoch-making investigations of the United States Army Commission composed of Walter Reed, James Carroll, Aristides Agramonte, and Jesse W. Lazear (1901), established the truth, that this disease, like malaria, is carried from one infected person to another through the agency of a mosquito. Finley, in 1881, was the first positively to assert that the mosquito was the transmitter of the disease. He was, however, unable to prove his theory, and it remained for the commission conclusively to show that a distinct species of mosquito carried the infection.

The work of the American commission was fully corroborated by the French commission and by other workers.

The principal facts established by the commission have been summed up by Goldberger as follows:

1. Yellow fever is transmitted, under natural conditions, only by the bite of a mosquito (*Aedes calopus*) that at least twelve days before has fed on the blood of a person sick with this disease during the first three days of his illness.

2. Yellow fever can be produced in man under artificial conditions by the subcutaneous injection of blood taken from the general circulation of a person sick with this disease during the first three days of his illness.

3. Yellow fever is not conveyed by fomites.

4. *Bacillus icteroides* Sanarelli stands in no causative relation to yellow fever.

Though the specific parasite remains yet undiscovered, facts have been brought out by these studies which give some idea of its character.

1. It seems to require two hosts (a mammal and an arthropod) for the completion of its life cycle (analogies, *Plasmodium malariae*, *Babesia bigemina*).

2. There is a definite time after the bite of the mosquito before the blood of the person bitten becomes infective (average five days), and a definite time that the blood remains infective (three days).

3. The blood during these three days is still infective after passing through the finest-grained porcelain filters (Chamberland B and F).

4. The blood loses its virulence quickly (forty-eight hours) when exposed to the air at a temperature of 24° to 30° C. When protected from the air by oil and kept at the same temperature it remained virulent longer (five to eight days). Heated for five minutes at 55° C. it becomes non-virulent.

5. The bite of an infected mosquito does not become infectious until twelve days (at a temperature of 31° C.) after it has bitten the first patient.

The cause of the disease still remains undiscovered, notwithstanding much study of human blood and other tissues and of infected mosquitoes. The infective blood filtrates show nothing with the dark-field illumination except small dancing granules similar to those found in healthy persons.

Certain facts relating to the disease seem to point to protozoa as the cause; for instance, the necessity for a second host and the long incubation time required before that host becomes infective after biting a yellow-fever patient.

The bodies described by Seidelin under the name *paraplasma* have been shown by Agramonte to be tissue changes.

The higher monkeys seem to be susceptible, though no complete experiments have been made with them.

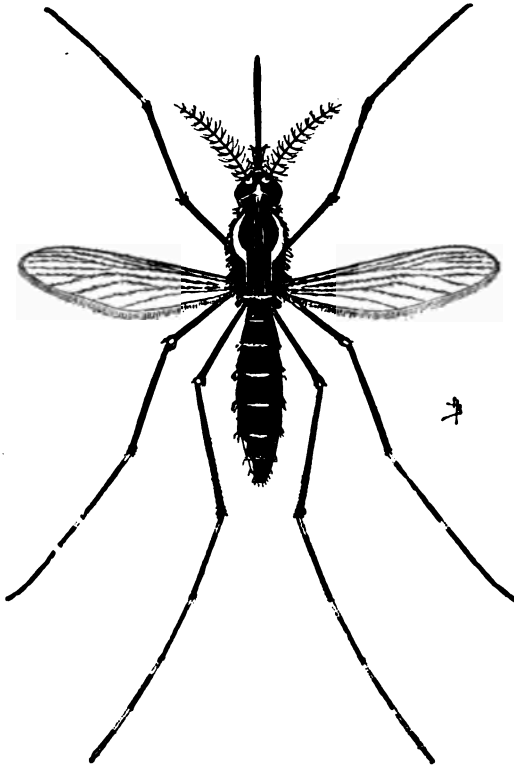


FIG. 198.—The yellow-fever mosquito (*Aedes calopus*). Adult female. Much enlarged. (Howard.)

**The Yellow-fever Mosquito** (Figs. 198-203).—The name *Stegomyia* for this small tropical mosquito was suggested by the English entomologist Theobald, who separated this genus from the genus *Culex*, with which it was formerly classed. It was first given the specific name *Fasciata*, but Blanchard proved that this had already been used and the name *Calopus* (Meigen, 1818) was found to be the proper one. Later the genus *Stegomyia* was shown to be invalid and the organism now goes by the name *Aedes calopus* Meigen. Salient characteristics of *Aedes* are: (1) The palpi in the male are as long or nearly as long, as the proboscis; in the female the palpi are uniformly less than one-half

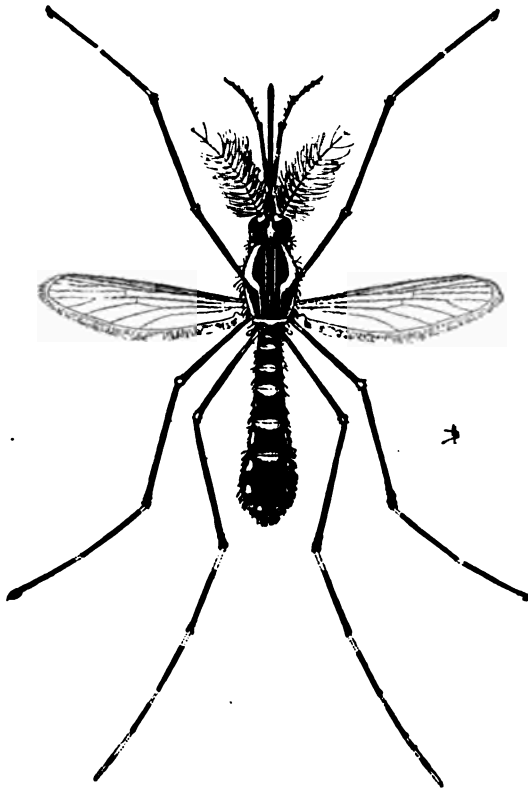


FIG. 199.—The yellow-fever mosquito. Adult male. Much enlarged. (Howard.)



FIG. 200.—The yellow-fever mosquito. Adult female, side view. Much enlarged. (Howard.)



FIG. 201.—The yellow-fever mosquito. Egg. Greatly enlarged. (Howard.)

as long; (2) the legs are destitute of erect scales; and are alternately banded white and black; (3) the thorax is marked with lines of silvery scales. *Aedes calopus* is spread over a wide range of territory, embracing many varieties of climate and natural conditions. It has been found as far north as Charleston, S. C., and as far south as Rio de la Plata. There is no reason to believe that it may not be present at some time or other in any of the intermediate countries. In the United States specimens of *Aedes calopus* have been captured in Georgia, Louisiana, South Carolina, and eastern Texas. The island of Cuba is

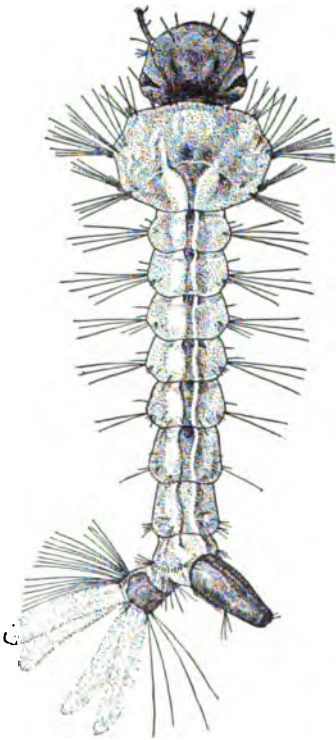


FIG. 202.—The yellow-fever mosquito.  
Larva. Much enlarged. (Howard.)

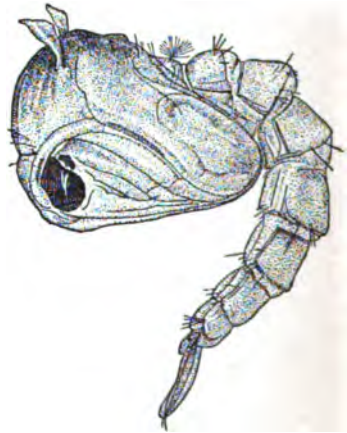


FIG. 203.—The yellow-fever mosquito.  
Pupa. Much enlarged. (Howard.)

overrun with this insect. The fact that *Aedes calopus* has been known to exist at various times in Spain and other European countries may account for the spread of yellow fever which has occurred there once or twice in former times; the same may be said of the country farther north in the United States, where *Aedes calopus* has not yet been reported, but which have suffered from invasions of yellow fever.

The adult mosquito is so small that mosquito netting of 19 or 20 strands or meshes to the inch is required to keep the insect from entering a place.

Brackish water is unsuited for the development of *Aedes* larvæ. The species *Aedes calopus* seems to select any deposit of water which is comparatively clean. The defective drains along the eaves of tile roofs are a favorite breeding place in Havana and its suburbs; indoors they find an excellent medium in the water of cups of tin or china into which the legs of tables are usually thrust to protect the contents from the invasion of ants, a veritable pest in tropical countries. The same may be said of shallow traps, where the water is not frequently disturbed.

Like other *Culicidæ*, it prefers to lay at night. It is eminently a town insect, seldom breeding far outside of the city limits. Agramonte never found *Aedes calopus* resting under bushes, in open fields, or in the woods; this fact explains the well-founded opinion that yellow fever is a domiciliary infection.

The question of hibernation in the larval stage is important. Agramonte failed to get larvæ that could resist freezing temperature, and found that in the case of *Aedes calopus* this degree of cold was invariably fatal.

The possibility of their being capable of life outside their natural element must also be considered from an epidemiological point of view. The dry season in the countries where this species seems to abound is never so prolonged as completely to dry up the usual breeding places. Experimentally, adult larvæ removed from the water and placed overnight upon moist filter paper could not be revived the following morning.

The question of the life period of the female insect is of the greatest importance when we come to consider the apparently long interval which at times has occurred between the stamping out of an epidemic of yellow fever and its new outbreak without introduction of new cases. The fact is that *Aedes calopus* is a long-lived insect; one individual was kept by Agramonte in a jar through March and April into May, in all for seventy-six days after hatching in the laboratory.

These mosquitoes bite principally in the late afternoon, though they may be incited to take blood at any hour of the day. They are abundant from March to September, and even in November Agramonte was able to capture them at will in his office and laboratory.

The mosquito is generally believed to be incapable of long flight unless very materially assisted by the wind. At any rate, the close study of the spread of infection of yellow fever shows that the tendency is for it to remain restricted within very limited areas, and that whenever it has travelled far beyond this, the means afforded (railway cars, vessels, etc.) have been other than the natural flight of the insect.

Experiments have demonstrated that not all mosquitoes which bite a yellow-fever patient become infected, but that of several which bite at the same time some may fail either to get the parasite or to allow its later development in their body. This condition is similar to that seen in *Anopheles*, with regard to malaria.

The question as to the length of time infected mosquitoes remain dangerous to the non-immune community cannot be definitely answered



at present; there is good presumptive evidence that the mosquito may harbor the parasite through the winter and be enabled to transmit in the spring an infection acquired in the fall. There is reason to believe that the mosquito, once infected, can transmit the disease at any time during the remainder of its life. Freezing temperature, however, quickly kills the insect.

Carrying out preventive measures based on the knowledge gained by the splendid work of the American Army Commission, yellow fever has been practically wiped out of Cuba, the Isthmian Canal Zone, and other infected areas.

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# PART III.

## APPLIED MICROBIOLOGY.

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### CHAPTER XLVI.

#### THE PRACTICAL APPLICATION OF BACTERIAL VACCINES.

THE practical application of bacterial vaccines for prophylactic purposes developed as a result of the success of vaccination against smallpox and the Pasteur prophylaxis in rabies. The first successful application was that of Pasteur who immunized sheep against anthrax by the injection of attenuated cultures. The protection conferred by the injection of bacterial vaccines is associated with a rise in the antibody content of the blood, and this increase has been assumed to be the basis of the protection. This increase may be only a part of the mechanism of protection, as the demonstrable antibodies disappear long before the immunity is lost. The view, however, that the protection was primarily due to the enhanced antibody content of the blood led to the therapeutic use of vaccines, with the hope that even during an infection they would stimulate further antibody production and thus be an aid in recovery.

The rational application of bacterial vaccines as specific prophylactic or therapeutic agents presupposes a correct bacteriological diagnosis, or at least a clinical diagnosis which warrants the deduction that the infection is due to a specific bacterium. It must be kept in mind that many of our bacterial names refer not to a single organism but rather to species in which the members of one subgroup frequently have little, if any, immunological relationship with members of other subgroups, or the members of subgroups may be completely heterogeneous as regards such relationship. On the diagnosis and a knowledge of immunological characteristics of the causative organism depends the selection of the vaccine and whether an autogenous or a stock vaccine is applicable.

**Polyvalent Vaccines.**—Many of the so-called polyvalent vaccines are not polyvalent in the sense that they contain representatives based on immunological knowledge. In most instances they are mixtures of many strains under a group name.

**Vaccines of Mixtures of Bacteria, so-called Mixed Vaccines.**—One type is a shotgun preparation advocated where a bacteriological diagnosis is absent. Another type is a mixture of the organisms usually

found associated in certain types of inflammation. Unfortunately the types found in such inflammation are usually members of heterogeneous groups so that the applicability of such stock vaccines as specific agents is more apparent than real. Mixed vaccines of specific types of bacilli for prophylactic purposes are discussed later.

**Types of Vaccines.—Killed Bacteria.**—This type of vaccine is the most commonly used. Heat is usually employed to kill the bacteria. The minimum temperature and time of exposure necessary to kill, should be used, as overheating may lower or destroy the antigenic value of the vaccine. Disinfectants such as carbolic, tricresol, etc., have been advised, but there is no definite evidence that they are superior to minimum heating. They are, however, often added as preservatives.

**Bacterial Extracts, Autolysates, Digested Bacteria.**—The object of such preparations is to hasten absorption or to bring into solution the toxic elements of the bacteria. In approximating these conditions, however, the reactions become severe or even serious and such preparations should be very cautiously employed.

**Live Bacteria.**—Their use is based on the fact that an unnatural portal of entry is associated with a local reaction only. The advantage would be that the unchanged bacteria should stimulate a higher immunity. There is always the possibility, however, that should they accidentally reach the normal portal of entry that disease might follow. The use of vaccines of bacteria attenuated in virulence by various methods is common in veterinary medicine.

**Sensitized Vaccines.—Living or Killed.**—The bacteria are treated with immune sera to lessen or avoid the local and general reaction, Besredka and others claiming that this will not interfere with their immunizing value. Larger and more frequent doses are thus possible and the sensitized bacteria are probably more quickly disintegrated and absorbed. The sensitized dead vaccines have been most commonly employed, and those left alive often die before use.

The superiority of sensitized over the ordinary killed vaccine has not been demonstrated. A similar procedure, viz., the simultaneous injection of vaccines and immune serum is employed in certain prophylactic procedures, where the bacterium employed gives rise to excessive reactions.

**Preparation of Bacterial Vaccines.**—Cultures are preferably grown on agar, although broth may be used. The growth after twenty-four to forty-eight hours, depending on the rapidity of growth of the organism, is washed off in a small amount of saline solution. This suspension should be well shaken to give an even distribution of bacteria and is then standardized before heating or the addition of preservatives. The Wright method is most commonly used for standardization.

A capillary pipette (see p. 220) is marked about one inch from the tip. The finger is pricked and blood drawn up to this mark; a bubble of air is then allowed to enter the tube and the bacterial suspension drawn up to the mark. The contents are then mixed on a slide and thin smears (as for blood) made and stained. On the under lens of the eye-piece a one-quarter-inch square is marked

out with a pencil, and using an oil-immersion objective the number of bacteria and red cells appearing in this square are counted separately. About fifty squares are counted and the average per square obtained. The number of red cells per cubic centimeter being known, the number of bacteria are obtained according to the following proportion: Number of red cells per square: number of bacteria per square :: 5000 (millions):  $\times$  (millions).

Other methods are employed as direct counting either by the Prescott method, in a counting chamber as for blood cells or platelets, centrifuging and determining the volume of bacteria or by determining the weight of the bacteria after evaporation and drying.

The suspension is heated to not over 55° C. for one hour unless the organism is not killed, and a preservative tricesol 0.3 per cent., carbolic acid 0.25 per cent. or lysol 0.25 per cent. added after making the necessary tests for sterility. For the American and English armies typhoid vaccine is only heated to 53° C. and the antiseptic relied on to finish sterilization. The vaccine is diluted for use with saline containing a preservative. The French add no antiseptic.

Sensitized vaccines are prepared by adding immune serum to the suspension and after several hours sedimenting the bacteria by means of the centrifuge and washing them free of serum with saline solution and suspending them finally in saline solution. Gay and Claypole employ alcohol-killed sensitized vaccines.

*Length of Potency of Vaccines.*—No definite knowledge is available. Until this is available it would seem advisable to limit the period to four or possibly six months.

**Therapeutic Application.**—*Theoretical Considerations.*—The use of bacterial vaccines in the course of an infection was primarily based on the conception that the infection was not a sufficient stimulus to the production of adequate curative antibodies. The additional stimulus of vaccines therefore would increase the antibody production and hasten cure. This conception is based on the idea of specific action. On this basis, localized lesions of a subacute or chronic character should be the type of case most benefited. Although this is, in general, the fact, the more acute and general types of infection are also benefited by vaccine therapy. It would seem that when the blood stream was invaded by bacteria, that all the tissues of the body would be stimulated to their maximum capacity of response. We must, however, distinguish three types of generalized invasion: first, where there is an initial blood invasion with secondary localization and the disappearance of the infective agent from the blood; second, acute local infections, with limited invasion, without multiplication in the blood (simple bacteremia); third, a septicemia, invasion and multiplication in the blood stream.

The first two show the ability of the body to limit the blood infection by a response of the protective forces of the body. This, however, may not be the maximum possible response and in these conditions vaccines do increase the antibody response, an example being typhoid fever. Furthermore, the injection of vaccines leads to a cellular response in the form of a polynuclear leukocytosis or an increase if already existent. It is evident, however, that injudicious dosage might overwhelm the

body and break down the ability to respond, with serious consequences to the patient. In the septicemia types, intravascular multiplication with no tendency to localization usually indicates the inability of the body adequately to respond, and little is to be expected from the further stimulus of vaccines. This is usually found to be the case.

Considering specific response to vaccines, one would not expect much benefit in acute self-limiting types of infection, as the antibody response requires several days to become marked.

Theoretically, if the antibodies cannot reach the focus of infection in sufficient concentration, vaccines would be of little help. This is probably a factor in the generally poor results obtained with infections of bone sinuses or cavities.

Vaccines are an aid, not a substitute for indicated surgical procedures, and the application of the latter should never be delayed. Incision, drainage and relief of tension, even if pus has not formed, are of curative value due partly to better circulation, also to the fact that drainage allows the exudation of fresh serum and cells, both of the utmost importance.

**Non-specific Response to Specific and Non-specific Vaccines.**—The occurrence of prompt curative response following the injection of not only specific but also of non-specific substances cannot be explained on the basis of specific antibody response. Such a response, especially following intravenous injection of non-specific substances would at first glance overturn all our ideas of the specific action of therapeutic vaccines. The recent methods of treating typhoid fever by intravenous inoculation is a good example. The injection of an appropriate amount of typhoid vaccine or even paratyphoid or colon vaccine or albumose, is followed by a chill and a rapid rise of temperature followed by a progressive fall, sweating, and marked subjective and general improvement. At first there is a decrease, then a sharp increase of the polynuclear leukocytes. In some cases the reaction develops into a recovery by crisis, in others the disease resumes its course and a second injection, giving a similar reaction, may or may not be followed by critical recovery. Similar curative reactions have been obtained in other infections. (See *Gonococcus*.)

As far as we know at present, the following factors enter into such curative reactions. The sharp leukocytic response is undoubtedly of value. Increased antibody production cannot occur even with specific vaccines; furthermore, vaccination during the incubation period of typhoid fever has little, if any, effect on the subsequent disease, which should be the case if the mere increase of antibodies were the important factor. On the other hand, the best results are obtained after the tenth day of the disease, that is, after response to infection has been fully established and the injection at this time is followed by a rapid increase of the antibodies in the blood. What apparently takes place is a stimulation of the hemopoietic organs (the probable source of antibodies) with the release and dispersion of antibodies already formed and the throwing out of polynuclear leukocytes. A further factor as pointed out by Job-

ling is the mobilization of non-specific ferments, the serum-protease, for instance, would act on the toxic products of the bacilli and reduce them to non-toxic products. The antiferment content of the serum is also influenced and may be a factor. Desensitization or a refractory condition of the cells to typhoid bacillus products has been suggested in explanation.

The chill and rise of temperature are important, as without these curative effects are not obtained. How much of the reaction is due to the injected protein alone, or to the dissolution products resulting from the sudden response of the body is not clear. Normal persons react similarly but larger doses are required and the reaction is less intense in the milder infections. Where there is a focal infection, reactions at the focus are undoubtedly of curative value. This is most evident in gonorrheal arthritis.

The ability to respond to non-specific or to specific substances run parallel, so that the data given as to selection of case apply equally.

Although non-specific substances are of curative value, the relative value of these as contrasted with specific vaccines, or to what degree the curative response to specific vaccines is of non-specific character, is still to be determined. Theoretically, the added specific response to vaccine of the autogenous type should be of value. Non-specific substances may raise the resistance to specific infection, but for prophylactic purposes specific vaccines must be employed.

The knowledge we have is no excuse for ignoring specificity nor for the general use of mixed and pseudospecific stock vaccines. It is unfortunate that many reports of beneficial results of vaccine therapy are valueless in this connection as the results are based on the assumption of specific action whereas the data given are no guarantee that such was the case and deductions are therefore impossible.

**Reaction to Vaccines.**—The reaction may be local, focal or general. As a rule, with appropriate dosage reactions should be slight or, at most, moderate. With intravenous therapy a reaction (see above) is essential.

**Dosage.**—The dosage given for prophylactic purposes is based on experience and should be closely adhered to. Children stand vaccines well, and full correction according to weight is unnecessary. For therapeutic purposes it is usually better to start with small doses and increase these until the maximum is reached, which is usually the reacting dose in the individual. The period between injections may be from twenty-four to seventy-two hours or longer. Continued injections increasing in amount frequently lead to cure even when no beneficial results are seen at first.

**Control of Dosage.**—The use of the opsonic index (see p. 217) has been abandoned, the dosage being based on reaction and clinical results.

**Negative Phase.**—Wright thought that there was an initial period of depression following vaccination. Unless the dose is large or recklessly administered, this need not be considered. Prophylactic vaccination can be carried out even though the person is exposed to infection and is

not injurious even during the period of incubation. At most it may accentuate the onset of the disease.

**Mode of Injection.**—This is usually subcutaneous. The intravenous method should not be undertaken unless one is conversant with the method and has a thorough understanding of its possible dangers. Intravascular agglutination with cerebral embolism, shock due to rapid dissolution of bacterial products and hemorrhage as in typhoid fever are the possible dangers. Several deaths can be directly attributed to this mode of injection. Intramuscular injections may also be used.

### PRACTICAL APPLICATION OF INDIVIDUAL VACCINES.

The following is a brief summary of the modes of administration and the results in various types of infections.

**Staphylococcus Infections.**—The best results have been obtained in chronic or recurrent types of infection, such as acne and furunculosis. Stock vaccines may be employed, but if failure is encountered autogenous vaccines should be tried. In the deep indurated types of acne other bacteria are probably important, as *B. acne*, and vaccines of the bacillus in doses of 2 to 20 million should be tried with the staphylococcus. In furunculosis the vaccine seems to be of more value in preventing new lesions than in the cure of the existing foci. Sycosis and other skin lesions with associated pustular lesions may be benefited by vaccines. Acute local lesions are probably little influenced, and in this and in the other conditions mentioned the usual modes of treatment should be employed. The greatest care should be taken to protect the healthy skin from discharges from the infected focus.

**Dosage.**—The more extensive the lesions the smaller should be the initial dose. From 100 to 1000 million is the average increase in dosage, though larger doses may be given.

**Streptococcus Infections.**—**Local Infection.**—Acute infections due to *Streptococcus pyogenes* are usually surgical conditions. In the subacute stage vaccines may be of help. Streptococci are found in many conditions, such as common colds, bronchitis, sinus involvement and mouth infection, but vaccines are of doubtful value. Puerperal infections are probably little affected by vaccines. The reported results in erysipelas are as variable as the disease itself. Erdman in an analysis of 800 cases could see no result.

**General Infections.**—The data are insufficient. There is a slight indication that immune serum followed by vaccines is of some value.

**Dosage.**—In severe or general infections an initial dose of 5 to 10 million, in more local lesions larger doses, may be given. The maximum is usually about 500 million.

**Pneumococcus Infections.**—**Pneumonia.**—Only a moderate number of cases have been treated under conditions of specific relationship of vaccine to the infecting pneumococcus. (See types under *Pneumococcus*.) Other favorable reports have been made without regard to this relationship. It may be that this disease can be favorably influenced by non-

specific therapy. At best the data at hand do not indicate any very marked results. As a prophylactic the vaccine has been used.

**Other Infections.**—See common colds, bronchitis, otitis, etc., p. 595.

**Dosage.**—Ten to 500 million.

**Gonococcus Infections.**—Urethritis is uninfluenced and the number of complications are not appreciably reduced. Vaginitis shows very little improvement, though this may be due, as Pearce points out, to immunological differences in the gonococci from those found in adult infections.

**Epididymitis and Prostatitis.**—Some cases have been benefited. *Pelvic infection* or *general infections* are little influenced. *Periarthritis* and similar conditions have been very successfully treated.

**Dosage.**—From 25 to 500 million is the usual dosage, though up to 1 billion have been given. The dose should be rather rapidly increased until some degree of reaction is elicited. Although a reaction of any extent is to be avoided, a mild focal or febrile reaction is not only of value in indicating the limits of dosage but is also of curative value.

**Intravenous Administration.**—Bruck and Sommer have advocated this method, and unusually good results are claimed not only in gonorrheal arthritis but also in epididymitis, prostatitis and even in urethritis. They used a preparation "Arthigon," 1 c.c. of which contains about 80 million gonococci. Müller and Weiss, and Miller and Lusk have had good results with non-specific substances.

**Meningococcus Infections.**—**Prophylaxis.**—Three injections at weekly intervals of 250, 500 million and 1 billion respectively give rise to considerable antibody production. The data as to the protective value are too limited to draw any conclusions.

**Therapeutic Application.**—In some cases where lumbar puncture and serum administration, although repeated frequently, has had little effect an autogenous vaccine may be of value. DuBois and Neal recommend an initial dose of 100 to 250 million, increased to 1 billion, giving the injections every two or three days.

**Micrococcus Catarrhalis.**—See under Common Colds, etc. Dose 10 to 500 million.

**Typhus Fever.**—The prophylactic use of vaccines is recommended by Plotz. As, however, he has not succeeded in producing immunity in experimental animals, it is well to reserve judgment on the favorable results reported in man. (See p. 454.)

**Typhoid Fever.**—**Prophylaxis.**—This method had its inception in the demonstration by Pfeiffer and Kolle and by Wright in 1896, that the injection of killed bacilli caused the production of the same antibodies as found in the blood of convalescent typhoid cases. In 1898 Wright inoculated 4000 men in India and Leishman supervised the inoculation of the British troops in the Boer War. In 1909 vaccination was started in the United States Army under the direction of Russell. The results in the present war have been astonishingly good and equally good results have been obtained in civil life.

**Selection of Vaccine.**—Various strains are used by the English, French and American Army medical men. The vaccine as advo-



cated by the United States Army officers is prepared from a strain (Rawlings) of known antigenic value and the vaccine is only heated to 53° C., relying on the added tricresol to kill any bacilli not killed by this degree of heating. Unfortunately, some of the vaccines marketed are relatively inactive, possibly due to preparation, heating, preservation, or too long a labelled period of potency or a combination of these factors. Sensitized vaccines have been strongly advocated by Besredka, Gay and others.

**Administration.**—A strong degree of immunity is only conferred by two large or three moderate doses. The army men receive 500 million, 1 billion and 10 billion bacteria. Successive Saturdays are most convenient and it is preferable to give the vaccine in the afternoon so that the reaction, if it occurs, will occur while the subject is abed. Injection should be subcutaneous at the insertion of the deltoid.

**Reaction.**—Usually only a local tender reddened area develops. In some cases it is more extensive and there may be some tenderness of the axillary nodes. Slight constitutional symptoms may develop but a severe general reaction is exceptional. The reaction is of no importance except for the discomfort and has no relation to the subsequent immunity. There is no reason why vaccination should not be done during exposure to infection.

**Results of Immunization.**—Among the many millions of men vaccinated during the present war there has been almost no typhoid fever. An excessive dose of infectious material may break down the protection which is only relative, but any extensive failure should raise a strong presumption that the vaccine employed was not satisfactory.

**Duration of Immunity.**—The degree of immunity decreases after two and a half years but even after four or five years the rate among the vaccinated may be only one-fourth that of the unvaccinated. Under conditions of constant exposure to infection associated with strain and privation as in the present war, the immunization should be repeated each year.

**Therapeutic Use.**—*Subcutaneous Injection.*—Watters has collected and analyzed 1120 cases. Seventy-one deaths occurred, that is, a mortality of 6.3 per cent. Probably 17 cases with 15 deaths could be omitted on the ground that they were moribund when treated or for other reasons. This would give a mortality of 5 per cent. The incidence of relapse was 6 per cent. in the cases where stated. Various-sized doses were employed by the different observers, and there is no correlation between dosage and mortality or relapse incidence. Many factors enter into the death-rate of this disease and the lowered rate cannot be directly attributed to the vaccines. In the different series also, the rate varied very widely. In general, the patients treated seemed brighter and the temperature averaged lower, and the febrile period was appreciably shortened. The more moderate dosage, 250 to 500 million would seem advisable.

*Intravenous Use.*—This method was introduced by Ickikawa in 1912. Several hundred cases have been treated up to date by various observers.

The reaction has been described (p. 588). About 50 per cent. quickly convalesce under this treatment. There is insufficient data to warrant deductions as to its influence on the death-rate. A number of cases have developed fatal hemorrhage from the bowel or elsewhere after inoculation and several cases have died shortly after injection; in 2 there was an associated pneumonia. Evidence of hemorrhage, pneumonia or cardiac disturbance are therefore contra-indications. The dosage and vaccines employed have differed widely. From 50 to 250 million is the average. Although Gay advocates the use of sensitized vaccines, no one vaccine seems better than another. (See also under Non-specific Vaccines.)

**Paratyphoid Infections.**—Paratyphoid fever is comparatively uncommon in this country, though under camp conditions it may become epidemic, as among the militia encamped on the Mexican border. It prevailed among the European troops before the use of vaccine.

**Prophylactic Vaccination.**—There seems no reason why this should not be as effective as with typhoid, although the epidemiological data does not allow of conclusions as yet. Vaccination seems absolutely necessary among troops, etc., as evidenced by our experiences as well as the conditions in the European war. It would not seem, however, that vaccination would give as complete protection against the food-poisoning types of infection where the dose of preformed toxin is large, although the immunity present may limit invasion by the bacilli.

Vaccines of the individual types may be employed in the doses recommended for typhoid. Mixed vaccines have been more commonly employed, thus first dose 500 million typhoid, 250 million each of Paratyphoid "A" and "B," second and third doses twice this amount. When time is not a factor it would be well to adhere to our present methods of typhoid prophylaxis and give the paratyphoid vaccine (mixed types) separately.<sup>1</sup> The European practice is to give them together.

**Bacillary Dysentery.**—Dysentery vaccines are highly toxic and Shiga has employed the simultaneous injection of vaccine and serum. The results are not wholly satisfactory, although the mortality among the vaccinated was lowered. The prevalence of different types of bacilli adds to the difficulties. It has been claimed that the vaccines are of value in the treatment of carriers.

**Plague.**—Prophylactic vaccination gives a relatively short period of immunity and is best undertaken during epidemics. The protection is only relative against bubonic plague but the mortality is also lowered. There is less protection against pneumonic infection. Haffkine advises 3 to 3.5 c.c. of his specially grown broth cultures, giving a second dose after eight to ten days. Kolle advises 2 mg. of the growth on agar. These amounts are fairly equivalent to 500 million bacilli.

**Cholera.**—Prophylactic vaccination affords considerable protection, but the mortality rate of those who become infected is only slightly

<sup>1</sup> The U. S. Army paratyphoid vaccine contains 1 billion *B. paratyphosus* A and 600 *B. paratyphosus* B to each cubic centimeter. The dosage is 0.5 c. c., 1 c. c. and 1 c. c. at 7- to 10-day intervals.

influenced. The reactions are frequently moderately severe. Live vaccines have also been employed.

**Combined Prophylactic Vaccines.**—Castellani is the main advocate. Combined vaccines against the endemic types of disease would be of advantage when time was a factor. The dosage is as with the typhoid bacilli. The addition of the cholera vaccine does appreciably increase the reaction.

**B. Pyocyaneus and B. Proteus.**—Usually encountered as secondary invaders, some benefit has been reported from the use of vaccines. The dosage is from 25 million to 1 billion.

**Glanders.**—There is some indication that vaccines are of value in subacute or chronic infections in man. The temperature should be carefully watched as the vaccine acts similarly to mallein. Dosage 10 to 100 million.

**B. Coli and Related Types.**—Genito-urinary infections seem, in some instances to be benefited, especially cystitis and possibly pyelitis after the acute symptoms have subsided. Vaccines may have some influence in diminishing the fever and discharge from sinuses after pelvic abscess, appendicitis, or cholecystitis. The mucoid *B. aërogenes* as well as intermediates resembling *B. paratyphosus* are frequently found in these types of infection. The dosage ranges from 25 to 500 million or more. Different strains vary in the degree of reaction produced.

**Atrophic Rhinitis and Rhinoscleroma.**—The etiology of the former is not settled, Perez claiming that the "*cocco-bacillus ozena*" is the cause. Vaccines of this organism with or without *B. ozenæ* have given suggestive results. Rhinoscleroma is possibly influenced by vaccines.

**Other Infections due to Encapsulated Bacilli.**—These types are encountered in infections of the respiratory tract or by extension in sinus, middle ear and mastoid. It is doubtful if vaccines are of any value, at least they cannot be applied during the acute stage.

**Dosage.**—The same as for *B. coli*. The initial dose of bacillus of Perez is 50 million to be increased until a focal reaction occurs.

**Pertussis.—Prophylaxis.**—Some protection is conferred but it is difficult to judge of the degree with the data available. Hess vaccinated 244 children and 20 developed the disease, whereas of 80 equally exposed children 59 developed the disease. These results as well as those of Still and Luttinger warrant its application, especially as the procedure is innocuous.

**Therapeutic.**—Hess could see no influence on the disease even where both prophylactic and therapeutic vaccines were given. It is difficult to correlate these results with the reports of others that the number of paroxysms and the duration of the disease is lessened. In some instances a prompt amelioration has been reported, so prompt as not to be explained on the basis of specific antibody production. We have been treating two series of cases, one with pertussis vaccine, the other with a vaccine of *B. influenza* which, though similar culturally, differs completely immunologically. One vaccine shows results about as good as the other. It would almost seem as though we were dealing with a non-specific action on the mucous membrane condition.

**Dosage.**—For children over one year 500 million, 1 billion and 2 billion at two-day intervals is recommended. Children under one year receive half these doses. If, after several days, improvement is not marked, further injections may be given. Prophylactic injections are given every third day, the doses being 500 million, 2 billion and 3 billion respectively.

**Influenza Infections.**—No data are available concerning epidemic influenza. The presence of influenza bacilli in inflammations of the mucous membranes, accessory sinuses and conjunctiva is not necessarily an indication of their etiological importance. The value of mixed vaccines containing influenza bacilli in this condition is problematical. The use of autogenous vaccines where the evidence points strongly to their etiological importance has given at best only suggestive results.

**Dosage.**—Initial dose 10 million to 20 million, which can be increased to 200 million to 500 million.

**Tuberculosis** (see Tuberculin Therapy in chapter on Tuberculosis).—Vaccines of the secondary invaders, in pulmonary phthisis have been tried with only meagre results.

**Focal Infection and Systemic Disease.**—In general the conclusion seems warranted that vaccines are insufficient if the focus is not eradicated, and if eradicated, vaccines are not necessary. Each case is a problem in itself and a careful study of typical case reports is necessary to a comprehension of the subject. Streptococci and gonococci are the most frequent causative organisms.

**Miscellaneous Conditions.**—*Common Cold.*—The beneficial results both prophylactic and curative, should be viewed with scepticism. Little is known concerning the etiology of common colds. Some outbreaks are due to a filtrable virus, according to Kruse and Foster. Micrococcus catarrhalis, influenza bacilli, pneumococci, streptococci, B. segmentosus, etc., have been encountered as the predominating organism. The types encountered are usually members of heterogeneous groups and it is difficult to see how mixed stock vaccines can have any influence at least from the specific stand-point. Possibly inoculation of such vaccines may have some obscure non-specific protective or curative influence on the mucous membranes.

*Bronchitis and Chronic Respiratory Conditions.*—Autogenous vaccines of the predominating flora cause at most a slight amelioration in a few cases.

*Sinus and Middle-ear Infections.*—The treatment of subacute or chronic infections has given little result, possibly because of the anatomical conditions.

*Mouth Infections.*—The use of vaccines in pyorrhea is advocated by some observers but they agree that local treatment is necessary as well, whereas others find that local treatment alone is all that is necessary. The vaccines employed only represent a small part of the aërobic flora, and the dominant anaërobic fusiform bacilli and spirochetes are ignored. Whether vaccines influence the general symptoms which may be associated is another problem. (See Focal Infections.)

*Selection of Vaccine, Dosage, etc., in above Conditions.*—An autogenous vaccine based on a careful bacteriological examination is alone applicable. The stock vaccines widely advertised are, so far as our present knowledge goes, non-specific vaccines, even though the contained organisms bear the same names as those encountered in the inflammation. The dosage for the individual types has been given. Pro rata reductions should be made according to the number of types in the vaccines.

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## CHAPTER XLVII.

### THE PRACTICAL APPLICATIONS OF SERUM THERAPY.

THE advisability of using sera in any particular disease is influenced by a number of considerations. The primary one is whether a serum has been obtained and is available which contains antibodies of suitable kind and amount to neutralize the toxins or aid the body cells and ferments in destroying the microorganisms. Others almost equally important are whether the infections can be identified from clinical signs alone, or only after additional laboratory examinations, and whether the sera can be brought into contact with the toxin or organisms at the essential points, in the necessary concentration and within the required time. Practical serum therapeutics in the more important infections in which some results have been obtained will be considered in the light of the above considerations.

#### SERUM TREATMENT OF LOBAR PNEUMONIA.

This disease is so uniformly due to one organism that from the clinical signs alone the physician can almost assume a pneumococcus infection. This knowledge is not as valuable as it seems since it has become evident that the characteristics which define a pathogenic microorganism are frequently so broad as to include a number of strains, which from the view-point of immune sera are as distinct as microorganisms with wide cultural difference. The term "pneumococcus" as pointed out by Neufeld is one of these broad group names which cover a number of strains each one of which is unaffected by the antibodies produced through immunizing injections with the others.

A further extremely important differentiation between the types is that with our present methods some excite by their injection into suitable animals abundant antibodies, while others do not. The reasons for this we do not understand. The most recent and most thorough investigation concerning the value of the specific serum for pneumonias due to each type of organisms has been carried on at the Rockefeller Institute for Infectious Diseases by Cole and his associates and the present favorable outlook for treating a certain proportion of pneumonia cases is largely due to their work. (See chapter on Pneumococci.)

Cole's, Longcope's and Richardson's statistics of the frequency and the mortality of pneumonias due to the types are of great practical interest.

	Rockefeller Institute (P. H. Longcope).						Univ. Penn. Hospital, (Richardson).	
	Number.		Incidence, per cent.		Mortality, per cent.		No.	Mortality, per cent.
	Cole.	Longcope.	Cole.	Longcope.	Cole.	Longcope.		
I. . . . .	78	13	33	23	25.0	12.5	60	31
II. . . . .	75	11	32	21	29.0	72.7	39	20
III. . . . .	22	7	9	14	45.0	85.7	13	6
IV. . . . .	48	21	20	40	12.5	23.8	83	43
Other bacteria	14	..	6					12

It is noticed that about 30 per cent. of the cases of pneumonia and about one-third of the total deaths are caused by infections with type I.

These figures are very important because at present the results of serum injections follow their antibody content and seem favorable in type I infections, doubtful in type II and negative in type III and in type IV.

**Method of Administration.**—The experimental work in animals and the observation of cases has led to the general use of larger injections and the substitution of the intravenous for the subcutaneous method. The size of the patient and the strength of the serum in antibodies have not been considered in controlling the size of the dose, although all accept the fact that theoretically they should. The serum should be standardized, as in the case of antitoxins, 0.2 c.c. of a recent serum should protect against 100,000 f. d. of a very virulent type I pneumococcus. Serum should seldom be put out or used that contains less than half this strength.

Cole advises that 80 c.c. of serum diluted with 80 c.c. of salt solution be injected intravenously and repeated about every twelve hours until permanent improvement is noted. Usually he gives three to five doses.

The repetition of the dose every twelve hours is founded on the desire to give it sufficiently often. Cole states that the transferred antibodies tend to disappear after a dose of serum. This is due, in his opinion, to combination with the antigen in the body. In our own experimental work we have found that a single dose of 0.1 c.c. protects a mouse for the next four days from an injection of 1000 fatal doses of living pneumococci, and as late as the fifteenth day, from 100 fatal doses. The mouse differs from the sick person in not having an infected area.

In order that comparative results should be obtained, it is necessary to have all antipneumococcic sera labelled as to the strength and nature of antibodies and the date of the last potency test. The serum used should have been tested within three months, for it gradually deteriorates.

Cole recommends that the serum be only given in a case after the bacteriological test has shown the type. I believe, in severe cases a first dose of type I serum should be given as soon as possible and the later injections only after the bacteriological report. Except in hospitals Cole's plan means in most cases a delay of at least twenty-four hours and generally prevents the use of serum altogether.

It is understood that the severe cases which receive an injection of type I serum and from which no bacteriological report is received cannot be considered as having any value in forming an opinion of the effect of the treatment;

Thus if Dr. Longcope had treated all his cases with type I serum and had not identified the infecting type in each of the 22 deaths among his 52 patients he could have had no conception of the value of the serum, for only one of the deaths and 13 of the cases were due to the type I organism.

**The Results of Large Intravenous Injections of Specific Serum in Type I Infections.**—There is in about 30 per cent. of the cases an almost immediate more or less severe chill with a considerable rise of temperature which lasts for a short period. This occurs usually after the first but sometimes after the later serum injections. If the blood contained pneumococci, they disappear within twelve hours after the injection. The temperature usually rapidly falls after the initial rise to a point lower than before the rise and the symptoms, as a rule, improve sooner than the average untreated case of equal severity. The mortality up to the present time has been much less than in the untreated cases. Serum sickness with rashes, painful joints, swelling of lymph glands and other symptoms occur to a greater or less extent in about 50 per cent. of the cases during convalescence. The serum sickness, while it lasts, is very annoying, but not dangerous.

The fact that such really promising results have been obtained in cases due to type I leads us to hope that Wadsworth, Cole and others will succeed in their endeavors to get a therapeutically valuable serum for types II and III and possibly so concentrate the antibodies as to use a combined polyvalent serum.

#### SERUM TREATMENT OF EPIDEMIC MENINGITIS.

The intraspinal method of injecting serum from horses immunized to meningococci in meningitis introduced by Jochmann has been approved by all. The collective investigation carried out under Flexner's supervision practically settled its value, and if it were not for the somewhat discouraging reports received from England during 1915 it would not be necessary to more than allude to the value of the serum.

Several reports from England mention a mortality under serum treatment of different collections of cases of from 52 to 63 per cent. and several experienced men have advised against the use of the serum.

Should these results in any way cause us to change our favorable opinion?

There are undoubtedly certain epidemics in which an unusual proportion of the cases develop a thick tenacious exudate which greatly hinders the successful use of the serum. A number of these have been found at the New York State Quarantine Station. Again the sudden great foreign demand for the serum caught some of the manufacturers with a small supply and the attempt to replenish their stock caused them to bleed their horses too frequently and thus obtain a serum which was found at a later period to be poor in antibody content. Unless the strains used are properly selected the antibodies may not be suitable to combine with the strain producing the local epidemic.

That one or more of these explanations for most of the poor results are true is rendered certain by the fact that when potent serum was used in several thousand cases among the English troops in 1916 the



results were very good. Since 1910 the New York City Health Department bacteriological laboratory has treated all cases of epidemic meningitis applying to it. Our mortality in different years has varied from 21 to 34 per cent. Drs. Sophian, DuBois and Neal, who have had charge of the serum treatment for the laboratory, are absolutely convinced of the value of the treatment, not only from the clinical course but from the changes which take place in the spinal fluid as shown by the disappearance of the meningococci.

**Administration of the Serum.**—Dr. Neal finds that too often the private physician fails to repeat the injections sufficiently often. It is very rare, except in a case already convalescent, that it is correct to give daily injections for less than four days. If the organisms or symptoms do not disappear the injections of 10 to 25 c.c. of serum should be continued for many days. Fortunately lumbar puncture with removal of fluid is of value in the treatment. Unlike pneumonia, practically all cases of epidemic meningitis can be treated by a single polyvalent serum. The different strains do, indeed, differ, but they have group relationships and intravenous injections of suitably selected strains cause the horse to produce a serum capable of influencing almost all strains. This has been recently carefully investigated by Dr. Wollstein at the Rockefeller Institute. Every lot of serum should be tested for its complement-fixing, opsonic and agglutinating power until some better method be devised to estimate its value. Its agglutinating value should be tested especially in relation to its activity against various strains. The results of the tests should be stated on the package. An agreement should be reached to make a standard unit. The fluid removed from every case of suspected epidemic meningitis should be examined microscopically and culturally, for only in this way can a correct diagnosis be made.

**Directions for Use of Serum.**—The following directions are issued by the Research Laboratory of the New York City Board of Health.

Perform a lumbar puncture under aseptic precautions in the third or fourth lumbar space. A general anesthetic should never be used. In hypersensitive patients a local anesthetic may be advisable. Have the patient lying on the side with the back arched so that there will be the greatest possible distance between the spines of the vertebræ. Find the notch nearest a line connecting the crests of the ilia. Introduce the needle, preferably a Quinke needle, in the midline and push forward and a little upward. The distance the needle goes in depends upon the age of the patient and the muscular development. It varies from  $\frac{1}{2}$  to 3 inches. Allow the cerebrospinal fluid to flow out until the pressure is so reduced that only 3 or 4 drops come per minute. If the fluid is cloudy, inject the serum immediately.

The serum is warmed to body temperature and injected very slowly under the least possible pressure. A funnel and the tube arrangement allowing it to run in by gravity should be used. The barrel of an ordinary syringe may be used as a funnel. The rubber tubing should be  $\frac{1}{8}$  to  $\frac{1}{4}$  of an inch in diameter and long enough so that the funnel

can be raised 12 to 15 inches. In general, the average dose for an adult is 20 to 40 c.c. and for infants and children 2 to 20 c.c. The amount depends as much upon the quantity of cerebrospinal fluid withdrawn as upon the age. An infant will frequently stand 10 to 15 c.c. without difficulty. The dose should usually be at least 5 to 10 c.c. less than the amount of cerebrospinal fluid withdrawn. When serum apparently runs in freely after a dry tap, it is advisable to proceed very slowly and to watch the patient carefully for the slightest change in pulse and respiration. In cases with very thick exudate which will not flow through the needle, gentle suction with a syringe may be tried. If that fails, a little serum injected will sometimes start the flow. When possible, further injections are made only after bacteriological examination has determined the cause to be the meningococcus. The antimeningitis serum does no harm in meningitis due to other organisms.

In severe cases it is best to inject the serum every twelve hours until there is improvement. In moderate and mild cases it should be repeated each day for the first four days. Further administration depends upon the patient's general condition and the bacteriological examination of the fluid. Usually four to six injections are necessary, but as many as fifteen or more may have to be employed.

During or immediately after the injection of serum the respiration may entirely cease or the pulse may become very rapid and thready. Such an occurrence, while alarming, is not necessarily serious and is best treated by immediate withdrawal of some of the serum if the needle is still in place. If the needle has been withdrawn, or, if after some of the serum is removed the symptoms do not ameliorate, artificial respiration should be resorted to for the respiratory condition and adrenalin or other stimulants hypodermically for the heart.

The successful treatment of cases of meningitis must always depend upon experience and judgment. It cannot be reduced to a rule of thumb.

In all cases with meningeal symptoms a lumbar puncture should be done. No ill effects follow, on the contrary the relief of the pressure frequently produces beneficial results.

#### ANTISTREPTOCOCCIC SERA.

The same reasoning applies as with the antipneumococcic serum and the same dosage and method of administration.

Some of the strains recur frequently, others infrequently in infections. If we suspect that the infection is due to the hemolytic type of organism we can rightly give this type of serum with the hope of some good resulting.

In the viridans type of infection we have no evidence that any good is done by serum. This is probably due to the fact that each organism produces its own antibodies.

The organism should be identified as soon as possible and, if virulent in mice, tested against the serum in a mouse. Much further combined

clinical and laboratory investigation is required before a decision can be reached as to the value of the serum and the best dosage.

If it were not for serum sickness there would be no question that injections should be made early before the infection has advanced and the streptococci acquired a somewhat greater resistance to the specific effect of the serum antibodies and ferments.

The repeated local bathing of infected tissues with the serum seems to have a more beneficial action than that exercised by a non-specific serum.

### **THE SERUM TREATMENT OF BACILLARY DYSENTERY.**

The earlier opinion has been confirmed that bacillary dysentery alone occurs in cold and temperate climates while in hot climates both bacillary and amebic cases occur in about equal numbers. The idea that summer diarrheas were frequently due to the dysentery bacilli has been discarded. The bacilli may be present at times in small numbers in these cases, but they have too little part in the disease process to require specific treatment. The dysentery bacilli may be divided into dysentery bacilli and paradysentery bacilli or into different strains of a group. Like so many other bacteria causing inflammations of mucous membranes the organisms exciting dysentery belong to a variety of strains.

For sporadic cases and those occurring in the beginning of an outbreak, a polyvalent serum must be used. In later cases in an epidemic where the strain has been identified the monovalent serum is to be used if possible.

The serum in mild cases is given in doses of from 10 to 30 c.c. twice daily according to the size of the person. In severe cases as high as 100 c.c. two or three times in the twenty-four hours can be given and in desperate cases the serum has been given intravenously. If given intravenously, the serum must be warmed and given slowly. The doses are to be continued from day to day until permanent improvement is established. Injections are usually continued for two or three days. The majority of those who have used the serum confirm Shiga's original belief that the results are good. Our own experience in a number of severe cases is on the whole favorable. We believe it is unnecessary to use the serum in slight cases. In favorable cases within six to twelve hours the constitutional symptoms frequently improve. The abdominal pains are less, the mental condition is better and the pulse slower and stronger. There is frequently at this time some reduction in the number of stools, though they may be more copious and have increased sloughs. The serum on the market is not standardized and some of it is valueless. The necessity of using a polyvalent serum in most cases also lessens its value. In spite of these objections the use of the serum in severe cases is strongly indicated. It is very desirable that records be kept of the cases treated and the results reported.

Shiga believes the mortality to be reduced by the serum treatment

from around 35 per cent. to about 9 per cent. Some report even more striking results, while still others have met with disappointment. Undoubtedly the extent of mixed infections with other organisms, the possibility of some cases having been due to amebas and the use of inactive serum must be considered as being possibly the cause of the failure of the serum in these cases. Ruffer and Willmore showed that the Shiga type of serum had no effect on their cases which were due to P or Y type of paradysentery bacillus. Either a polyvalent serum or a suitable monovalent serum was found effective. The usual serum after-effects may develop.

#### **THE THERAPEUTIC USE OF HUMAN CONVALESCENT BLOOD HAVING SPECIFIC ANTIBODIES.**

Convalescent serum or whole convalescent blood has been used in the treatment of early toxic cases of scarlet fever both here and abroad and has given encouraging results in the limited number of cases observed thus far. Reiss and Jungman recommended the intravenous injection of 50 to 100 c.c. of pooled convalescent serum, while Zingher makes use of the intramuscular injection of whole convalescent blood, citrated or non-citrated, which he injects in quantities of 120 to 240 c.c. The blood causes no local inflammatory reactions in the muscles, and is rapidly absorbed. The convalescent serum or fresh whole blood is obtained from patients who are two or three weeks convalescent from scarlet fever. These donors should be free from syphilis and tuberculosis.

The effect of convalescent serum or whole blood in the uncomplicated early toxic cases of scarlet fever is seen in a critical drop in temperature, beginning about six hours after the injection and ending in from twenty-four to thirty-six hours; an early fading of the rash; improvement in the circulation and character of the pulse; and especially in the general condition and mental symptoms of the patient. Zingher reported the results obtained at the Willard Parker Hospital with intramuscular injections of whole convalescent blood in 15 very toxic cases of scarlet fever, selected out of a total of 900 admissions. A striking improvement was noted in 5 cases, improvement and final recovery in 6 more, while 4 patients died from various septic complications.

In the later septic cases, seen from the fifth to tenth day of disease, complicated by an extensive streptococcus exudate over fauces and tonsils, enlarged and tender cervical glands, a poor circulation and showing general septic temperature, fresh normal blood, injected in quantities of 120 to 240 c.c. and repeated if necessary in three or four days has shown very beneficial effects in some desperately ill cases. Fresh normal blood has no specific action in septic cases of scarlet fever, but it supplies definite nutritive, stimulating, and normal bactericidal substances.

### USE OF DIPHTHERIA ANTITOXIN IN TREATMENT AND IMMUNIZATION.

The antitoxin in the higher grades of globulin solution or serum is identical with that in the lower grades; there is simply more of it in each drop. In treatment, however, for the same amount of antitoxin we have to inject less foreign proteins with the higher grades, and therefore have somewhat less danger of rashes and other deleterious results. The amount of antitoxin required for immunization is 300 to 500 units for an infant, 500 to 1000 for an adult, and proportionately for those between these extremes. The larger doses are advised when the danger of infection is very great. After the observation of the use of antitoxin in the immunization of many thousand cases, we have absolute belief in its power to prevent an outbreak of diphtheria, when given in the amounts advised, for at least twelve days, and also of its almost complete harmlessness in the small doses required. When double the above quantities are given the immunity is prolonged on the average for about one week. If it is desired to prolong the immunity the antitoxin injection is repeated every ten days.

**Treatment.**—Although more than twenty years have elapsed since the introduction of diphtheria antitoxin in the treatment of diphtheria, good observers, although nearer together than at first, still differ in the amount which they believe should be injected and in the method of its administration. Before giving our own conclusions on the proper dosage, it is well to consider several important points upon which this dosage is founded.

The amount of toxin in any case of diphtheria is comparatively small. One hundred units of antitoxin which would neutralize fifty times the amount of toxin sufficient to kill a six-year-old child, would surely make harmless all the toxin present in the most malignant cases if it could gain access to it in time. If we gave antitoxin, therefore, as many suppose, simply in sufficient amount to neutralize the poison in the body of an infected person, comparatively small amounts would be injected, but we have to give very much more than this because of the time it requires for much of the antitoxin to reach the toxin. This can be brought into direct contact with the toxin only by being absorbed into the blood and then passing through the capillary walls to the tissue fluids and cells. The greater the quantity of antitoxin that is in the blood, the greater will be the speed that an appreciable amount will pass to the tissues. The combined endeavor of the clinical observer and the laboratory worker is to find the suitable dose which will give a sufficient concentration in the blood to neutralize, as quickly as necessary, the toxin in the tissues. In the laboratory we can test the amount of antitoxin which is absorbed into the blood from any given dose and the amount which passes out to the tissues, while the clinical observer can note the changes which take place as he watches the case after antitoxin treatment.

It is naturally a matter of great importance as to how the antitoxin

is administered. When given subcutaneously, the swelling caused by its injection rapidly disappears by the absorption of the water, but the globulins and antitoxin remain behind in the tissues because

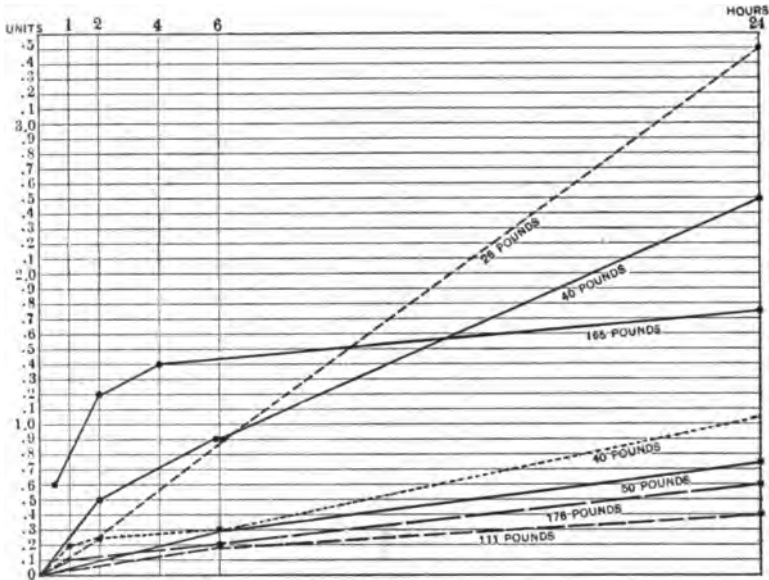


FIG. 204.—Amount of antitoxin in 1 c.c. of serum from persons, at different intervals of time, after a single *subcutaneous* injection of 10,000 units.

of the slow absorption of proteins. By testing many patients, it has been found that it takes twenty-four hours for the major part of the antitoxin to be absorbed by the blood from the subcutaneous tissues

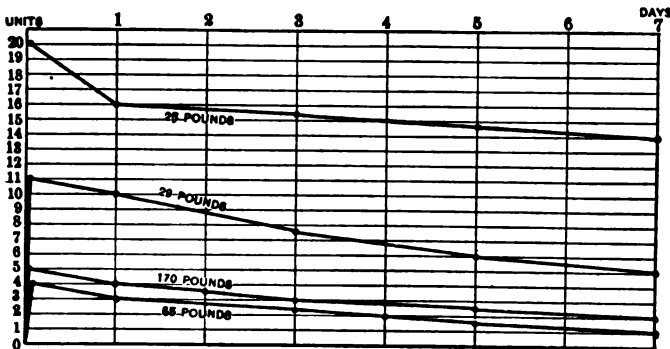


FIG. 205.—Amount of antitoxin in 1 c.c. of serum from persons, at different intervals of time, after a single *intravenous* injection of 10,000 units.

and some twelve hours from the muscles. For its total absorption it requires two or three days. Through the use of the Schick test, it has been determined that an injection of antitoxin given intravenously

passes out to the tissue fluids about ten times as rapidly as when the dose is given subcutaneously and four times, as when given intramuscularly. A unit gives most effect when given intravenously and least when given subcutaneously. If it were not for the fact that it is more difficult to give it intravenously and also that sharper serum reactions occur, the intravenous method would be the only one used. Another matter which is of importance is the size of the individual treated. It is self-evident that if a child weighing twenty pounds is injected with 10,000 units, it would, on the average, have in its blood five times as much antitoxin per cubic centimeter as a person receiving the same amount who weighs 100 pounds. The influence of weight on the dose is, however, largely neutralized by the fact that diphtheria in the child is generally more dangerous than in the adult. Every minute

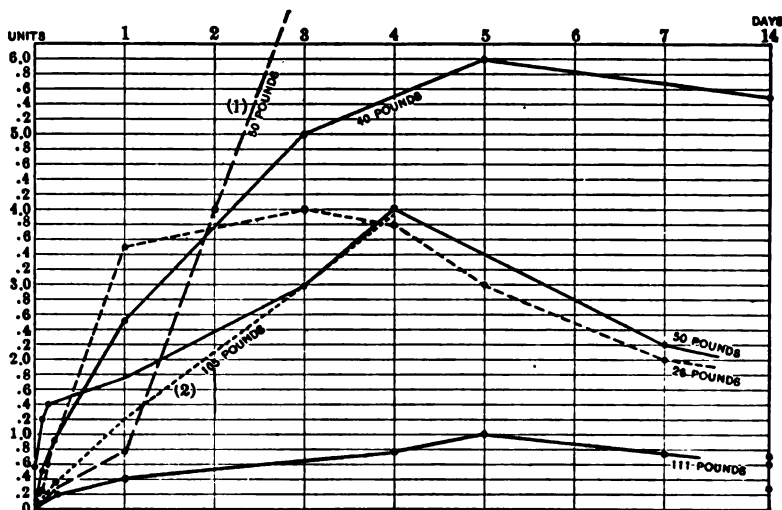


FIG. 206.—Amount of antitoxin in 1 c.c. of serum from persons, at different intervals of time (days), after a single subcutaneous injection of 10,000 units.

of delay in the neutralization of the toxin in a severe case is of importance, but in a mild case, where dangerous poisoning is still remote, slight delay makes little difference. Infants and children are especially liable to laryngeal diphtheria, so that every case in a child presents a certain gravity which the adult does not present.

The last point to be considered is whether a single or a multiple dose should be given. It must be realized that antitoxin has no effect whatever on injury which has already taken place. It is as useless as water on the ashes of a burned-out building. If the toxin is permanently united with the cell substance, antitoxin is no longer of any service. It is the early and sufficient dose which is important. When we give a divided dose, we simply get the effect of the first portion during the interval before the giving of the second dose. If the second dose had been given with the first, we would have had its effect added,

and so an insufficient dose made adequate. When the first dose has been of a sufficient size, the second and third injections, though harmless, are absolutely useless. The holding back of a part of the first dose so as to give it later, simply delays its action to a time when it cannot have much, if any, effect.

For the last three years, we have used in the hospitals for contagious diseases only a single dose of antitoxin, which in mild cases, has been given subcutaneously; in moderate cases subcutaneously or intramuscularly; and in severe cases, intravenously or intravenously and intramuscularly. After twenty years of experience in treatment and animal experimentation and consultation with physicians in New York and elsewhere, the following dosage, which is that adopted by the Health Departments of the City and State of New York, is advised.

#### DOSAGE OF UNITS OF ANTITOXIN IN DIPHTHERIA.

##### SINGLE DOSE ONLY.

	Infant, 10 to 30 pounds (under two years)			
Mild.	Moderate.	Severe.		Malignant.
2000	3,000	5,000		
3000	5,000	10,000		10,000
	Child, 30 to 90 pounds (under 15 years)			
3000	4,000	10,000		10,000
4000	10,000	15,000		20,000
	Adults, 90 pounds and over.			
3000	5,000	10,000		15,000
5000	10,000	20,000		40,000

##### METHOD OF ADMINISTRATION.

	Intramuscular or			
Subcutaneous	Intramuscular	½ Intravenous	½ Intravenous	
or	or	and	and	
intramuscular	subcutaneous	½ intramuscular	½ intramuscular	
		or	or	
		subcutaneous	subcutaneous	

The above amounts are sufficiently large, and I think no appreciable advantage would be obtained by increasing them. Very much smaller doses are still able to do great good, as the general blood current soon becomes antitoxic and blocks any further passage of toxin from the diseased tissues to other portions of the body. The feebly antitoxic plasma gradually permeates the body. There will be, however, more delay in the improvement of the local process, for the neutralization of the concentrated toxin in the diseased tissues will be slower. The exudate or pseudomembrane will continue to increase for some hours after the complete neutralization of the toxin because the injury to the tissue takes time to manifest itself.

**Determination of the Presence of Antitoxin in the Living Body and Results of Toxin-antitoxin Immunization.**—See pp. 312-315.

**Danger in Giving Injections of Antitoxin.**—About 1 in 10,000 persons develop, within a few minutes after an injection of serum, alarming symptoms. About 1 in 50,000 of the injected die. About 30 deaths in all have been reported. In 140,000 persons injected by New York



City Health Department Inspectors there have been 2 deaths due to serum. About the same proportion is reported from Boston. The persons suffering severe symptoms have usually been subject to asthma, while the fatal cases usually have the pathological changes known as status lymphaticus. A few of these rare cases die almost instantly. As a rule, when death occurs it takes place within a few minutes after the development of symptoms. Usually the respiratory rather than the circulatory centre seems to be affected. Persons who have not reacted badly to a first injection do not need to fear a second. So far as known all fatal results have followed the first injection.

**Results from the Use of Antitoxic Globulin Solution.**—The curative effect proved to be identical with that of the whole serum. Our tests showed clearly that not only the toxin, but also the poisons produced in the animal by injections with virulent bacilli are neutralized as completely by the globulin solution as by the antitoxic serum from which it is separated. The injections of the globulin solution were found to be followed by decidedly less severe rashes than the whole serum, and it was especially noted that there were very few who had any constitutional disturbances even when the development of the rashes did occur.

The following comparative table gives a summary of the constitutional and local reactions obtained in the treatment of 50 cases of diphtheria in young children, with a lot of antitoxic serum received from three horses which was found to produce an excessive amount of disturbance, and of an equal number of similar cases treated with a solution of the antitoxic globulins derived from a portion of the same lot of serum is as follows:

	Children treated with the whole serum.	Children who were treated with the antitoxic globulins.
Marked constitutional symptoms accompanied by a severe and persistent rash . . . . .	28 per cent.	0 per cent.
Moderate constitutional symptoms accom- panied by a well-developed erythema or urticaria . . . . .	18 per cent.	4 per cent.
Very slight constitutional disturbance accom- panied by a more or less general urticaria or erythema . . . . .	20 per cent.	8 per cent.
No appreciable constitutional disturbance but more or less general urticaria or erythema . .	4 per cent.	34 per cent.
No appreciable deleterious after-effects what- ever . . . . .	30 per cent.	54 per cent.

#### DURATION OF RASHES.

Days	1	2	3	4	5	6	7	8	Totals
Antitoxic globulin cases . . . . .	5	7	5	2	3	..	1	..	23
Whole serum cases . . . . .	1	4	10	1	10	3	2	5	36

The concentration of antitoxin made possible by the elimination of the non-antitoxic substances is not only a convenience but is of a distinct importance, as it tends to encourage large doses. Some producers, however, supply a product which is so rich in protein as to be almost semisolid. This is not quite so well absorbed as the less concentrated product. The total solids in the globulin solution should not be more than twice those in the serum.

The antitoxic globulin solution tends to become slightly cloudy when kept at moderate or high temperatures. This does not interfere with its potency. Substances such as solutions of carbolic acid and tricresol precipitate it but not in the quantity usually employed as preservatives.

### **TETANUS ANTITOXIN IN TREATMENT AND IMMUNIZATION.**

While tetanus antitoxin has proved most efficacious in the prevention of tetanus, its employment as a curative agent has been much less successful. This failure to produce more uniformly good results is due chiefly to its too late administration. Insufficient dosage and the use of the subcutaneous method have also been important factors. While the subcutaneous use of antitoxin, which at first was the usual method employed, may prove of value in large doses given within the first few hours after the onset of symptoms, nevertheless it frequently fails because of slow absorption and the time required to reach the tissues of the central nervous system. In the light of our present knowledge, this method can be justified only by an inability of the physician, for one reason or another, to give an intravenous or an intraspinal injection. The latter method is being more and more recommended, and when employed before the disease has made too much headway has given much better results than the subcutaneous or even the intravenous.

From time to time since 1903 single cases or small series of cases have been reported in which the antitoxin has been given intraspinally. Thus Neugebauer<sup>1</sup> in 1905 reported 43 cases from Continental and American sources with a mortality of 22, or 51 per cent., and also 3 patients treated by him of whom 2 recovered. Many of these patients received also intravenous or subcutaneous injections or both. The results seemed to some observers to be better than when other methods were used without intraspinal dosage, but on the whole, this method of treatment has not made much headway. Experimental proof of the greater value of the intraspinal method has not until very recently been at all convincing. Permin, of Copenhagen, working on rabbits and dogs, showed that local tetanus could be prevented by the simultaneous injection of tetanus toxin intramuscularly and a dose of antitoxin given in the spine, whereas with the same dose of antitoxin given intravenously, local tetanus occurred. Four hours after the giving of the toxin alone neither method of preventing the occurrence of local tetanus was efficacious, and when nine hours were allowed to elapse between the giving of toxin and antitoxin, the animals could not be saved.

The following series of experiments were undertaken by Nicoll and us to determine to what extent tetanus antitoxin given in the spine has greater curative power, when the disease is actually established, than when given in the circulation. The comparative ineffectiveness of subcutaneous injections of tetanus antitoxin in developed cases is inci-

<sup>1</sup> Wiener klin. Wchnschr., 1905, xviii, 450.

dently brought out. The use of guinea-pigs for this purpose has not been recorded so far as we know. They possess, however, a marked susceptibility to tetanus together with comparative freedom from intercurrent diseases. The toxin in each case was inoculated intramuscularly into the thigh.

ADMINISTRATION AND RESULT OF TETANUS ANTITOXIN.

No.	Weight, gms.	Condition of leg.	Method.	Amount in units.	Result.
116	290	Fairly stiff	Control	...	D 3 days
42	310	Fairly stiff	Control	...	D 3 days
296	250	Slightly stiff	Heart	100	D 8 days
227	275	Fairly stiff	Heart	100	D 4 days
399	300	Fairly stiff	Heart	100	D 5 days
216	255	Slightly stiff	Nerve	200	D 4 days
287	255	Fairly stiff	Nerve	200	D 3 days
289	280	Fairly stiff	Nerve	200	D 3 days
306	285	Slightly stiff	Nerve	200	D 3 days
59	255	Stiff	Spine	10	Discharged; normal
304	275	Fairly stiff	Spine	10	Discharged; drags leg
321	320	Fairly stiff	Spine	10	Discharged; drags leg

SIX GIVEN ANTITOXIN TWENTY-TWO AND A HALF TO TWENTY-THREE HOURS  
AFTER INOCULATION OF TOXIN.

102	300	Stiff	H.	100	D 5 days
10	325	Stiff	H.	200	D 4 days
272	350	Stiff	H.	200	D 4 days
263	285	Stiff	Sp.	50	Discharged; drags leg
123	325	Stiff	Sp.	50	D 5 days
294	350	Stiff	Sp.	50	Discharged; drags leg

This would seem absolutely conclusive of the superiority of the intraspinal method of giving antitoxin over the intravenous method. It will be noted that only those animals receiving the antitoxin in the spine were able to survive. This result is the more striking since the amount of antitoxin given them was only a fractional part of that given in the circulation. An attempt was made to give the antitoxin to four guinea-pigs intraneurally. Under an anesthetic the sciatic nerve of the affected limb was cut down upon and freed from the surrounding tissues, as much antitoxin as could be introduced into the nerve sheath injected, and the remainder intramuscularly directly along the course of the nerve. Owing to the small caliber of the nerve, most of the antitoxin passed to the deep intramuscular tissues. The animals lived no longer than those receiving subcutaneous injections.

In all of these experiments no guinea-pigs were discharged from observation until there was absolutely satisfactory evidence that the disease had long ceased to make any progress. While a number of the animals, perhaps most, on being discharged exhibited more or less stiffness in the inoculated leg lasting for weeks or even months, they were nevertheless in the best of health in other respects. This condi-

tion appears to be identical with that seen in human tetanus, in which the stiffness in different groups of muscles is often very prolonged even when the patients are perfectly convalescent.

Since beginning this work we have obtained records of twenty-four consecutive clinical cases of tetanus in which an intraspinal injection of antitoxin was given and eighteen patients recovered. In all of them in addition to the antitoxin used intraspinally, larger amounts were also given by other methods.

On experimental and clinical grounds the following recommendation for the treatment of tetanus with antitoxin would seem to be amply justified: In every case strongly suspected of being tetanus, from three to five thousand units of tetanus antitoxin should be given at the first possible moment intraspinally, slowly by gravity, and always, if possible, under an anesthetic. In order to insure its thorough dissemination throughout the spinal meninges the antitoxin should be diluted if necessary, to a volume of from 3 to 10 c.c. or more, according to the patient's age. When fluid is drawn off previously to the giving of the antitoxin, an amount of the latter somewhat less than that of the fluid withdrawn should be given. A number of cases of "dry tap" have been observed in the disease by those so expert in spinal puncture as to leave no room for doubt that the canal was properly entered. In such cases only a small amount of tetanus antitoxin should be injected (from 3 to 5 c.c.). In brief, tetanus antitoxin should be used in precisely the same way as antimeningitis serum.

**Results of the Use of Antitoxin for Immunization.**—The striking results which have been obtained, both in human and in veterinary practice, with the prophylactic injection of tetanus antitoxin, would seem to warrant the treating of patients with immunizing doses of serum—at least in neighborhoods where tetanus is not uncommon—when the lacerated and dirty condition of their wounds may indicate the possibility of a tetanus infection.

Splendid results have followed this practice in many places. It is the custom at many dispensaries in New York City and elsewhere to immunize all Fourth-of-July wounds by injecting 1000 units. None of these have ever developed tetanus. Even the few cases of human tetanus reported as occurring after single injections of antitoxin prove the value of immunizing injections, for the mortality was low. They teach, however, that where tetanus infection is suspected the antitoxic serum should be given a second and even a third time at intervals of seven days. In the European armies it is compulsory to give a serum injection to every wounded soldier at the first possible moment after the injury. A second injection is given ten days later. Since these regulations were adopted there have been almost no cases of tetanus develop.

## CHAPTER XLVIII.

### THE BACTERIOLOGICAL EXAMINATION OF WATER, AIR, AND SOIL. THE CONTAMINATION AND PURIFICATION OF WATER. THE DISPOSAL OF SEWAGE.

#### EXAMINATION OF WATER.

THE bacteriological examination of water is undertaken for the purpose of discovering whether any pathogenic bacteria are liable to be present. The determination of the number of bacteria in water was for a time considered of great importance, then it fell into disrepute, and the attempt was made to isolate the specific germs of diseases which were thought to be water-borne. At first these attempts seemed very successful in that supposed typhoid bacilli and cholera spirilla were found. Further study revealed the fact that there were common water and intestinal bacteria which were so closely allied to the above forms that the tests applied did not separate them. When proper identification was carried out the very great majority of the suspected organisms were found to be non-pathogenic. The improbability of getting typhoid bacilli from suspected water except under unusually favorable conditions caused a return to the estimation of the number of bacteria in water and above all to the estimation of the number of intestinal bacteria. It is known that the group of colon bacilli have a somewhat longer existence than the typhoid bacilli, and as the colon bacilli come chiefly or wholly from the intestinal passages of men and animals, it was fair to assume that typhoid bacilli, dysentery or other pathogenic bacteria could not occur from fecal pollution without the presence of the colon bacillus except in rare cases.

During the past few years the attention of sanitarians has been seriously devoted to the interpretation of the presence of smaller or larger numbers of colon bacilli in water, until at present, upon the quantitative analysis (measuring, within certain limits, decomposing organic matter) and the colon test (indicating more specifically pollution derived from intestinal discharges of man or animals) the bacteriological analysis of water is based. The determination of the number of bacteria is also of value.

**Technic for Quantitative Analysis.**—The utmost care is necessary to get reliable results. A speck of dust, a contaminated dish, a delay of a few hours, an improperly sterilized agar or gelatin, a too high or too low temperature, may introduce an error or variation in results which would make a reliable test impossible.

**Collection of Samples.**—The small sample taken must represent the whole from which it was drawn. If a brook water, it must be taken some distance from the bank; if from a tap, the water in the pipes

must first be run off, for otherwise the effect of metallic substances will invalidate the results; if from lake or pond, the surface scum or bottom mud must be avoided, but may be examined separately. The utensils by which the water is taken should be of a good quality of glass, clean and sterile. From a brook the water can be taken directly into a bottle, the stopper being removed while it fills, avoiding the surface film and its attending excessive numbers of bacteria; from a river or pond it can be taken from the bow of a small boat, or from a bottle properly fastened on the end of a pole so as to avoid contamination; from a well a special apparatus has been devised by Abbott, where a bottle with a leaded bottom is so held that when lowered to the proper depth a jerk will remove the cork and allow the bottle to fill. The same device or another accomplishing the same purpose can be rigged up readily by anyone. The sample of water should be tested as soon as possible, for the bacteria immediately begin to increase or decrease. In small bottles removed from the light, predatory microorganisms and many bacteria begin to increase, and among these are the members of the colon group. Thus, the Franklands record a case in which in a sample of well water kept during three days at a moderate temperature the bacteria increased from 7 to 495,000; while Jordan found that in a sample the bacteria in forty-eight hours fell from 535,000 to 54,500. In a sample we kept at room temperature the colon bacilli during twenty-four hours increased from 10 to 100 per c.c. The only safe way to prevent this increase is to plate and plant the water in fermentation tubes within a space of one or two hours or to keep it at a temperature under 5° C. (41° F.). If it cannot be kept cold, it is far better to make the cultures in the open field or in a house rather than to wait six to twelve hours for the conveniences and advantages of the laboratory.

The third matter of great importance is the adding of proper amounts of water to the broth in the fermentation tubes and the media for planting. Usually 1 c.c., 0.1 c.c., and 0.01 c.c. are added to the fermentation tubes and to 10 c.c. of the melted nutrient agar or gelatin. If possible, duplicate tests should always be made. When it is desired to know whether colon bacilli are present in larger amounts than 1 c.c., quantities as great as 10 to 100 c.c. can be added to bouillon, and then after a few hours 1 c.c. added to fermentation tubes. Less than twenty colonies and more than two hundred on a plate give inaccurate counts, the smaller number being too few to judge an average and the larger number interfering with each other. When as many as 10,000 colonies develop in the agar contained in one plate, it will be found that there will develop in a second plate containing but one-tenth the amount of water from 20 to 50 per cent. as many colonies. This shows that the crowding of the colonies had prevented the growth or caused a fusion of all but one-fifth to one-half of them.

The chemical composition of the medium on which the bacteria are grown affects the result of the analysis. Nutrient 1.5 per cent. agar gives slightly lower counts than gelatin, but on account of its con-

venience in summer and its greater uniformity it is being more and more generally used for routine quantitative work. A uniform standard is a necessity to secure comparability of results. (See Media for Water.) At best only a certain proportion of bacteria develop, and it is only important that our counts represent a section through the true bacterial flora which fairly represents the quick-growing sewage forms. Comparability is the vitally essential factor.

The temperature at which the bacteria develop is of great importance, and they should be protected from light. The access of oxygen which prevents the growths of anaërobes must also not be forgotten. As a rule, the plate cultures are developed at two temperatures, for four days at 20° to 21° C., and for twenty-four to forty-eight hours at incubator temperature. Some bacteria do not develop colonies in four days, but these are neglected. The number of bacteria growing at room temperature is usually much greater than those growing at 37° C. As all the intestinal groups of bacteria grow at body temperature, while many of the water types do not, some investigators believe it important to develop the bacteria at both temperatures so as to compare the results. We have not found this to be of any advantage when tests are also made for the colon group of bacilli.

The lactose broth, with indicator, is placed at 37° C. for growing the colon bacilli. The fermentation tubes not showing gas are recorded as negative and usually discarded. Those showing gas are suspected to contain colon bacilli. To a number of tubes containing melted litmus-lactose agar at about 44° C., are added 1, 0.1, and 0.01 loop of the culture fluid. Plates are poured and the whole placed in the incubator. The *Bacillus coli* ferments lactose and thus produces acid, so that if colon bacilli are present we have a number of red colonies on a blue field. Later, if many colon bacilli were present, the whole medium becomes acid. At forty-eight hours, on account of alkali being produced by the formation of  $\text{NH}_3$ , the blue may return. If after inspection red colonies are seen, four or five are picked and planted into lactose bouillon and other media. Litmus lactose agar is frequently used for the original plating of water samples, the absence or presence of acid-producing colonies being thus immediately noted. The results of these tests are all more or less presumptive evidence of the bacilli belonging or not belonging to the intestinal colon bacillus group. Unfortunately there are some types of bacilli growing in the soil which resemble them. If it is necessary to be more accurate, the colon-like cultures should be subjected to the Vosges reaction (page 125), and should be kept for one month at 20° C. in gelatin before a decision is made. Colon bacilli do not liquefy gelatin nor give the Vosges reaction (page 125). There are a few colon-like bacilli in the intestinal tract that give the Vosges reaction. For a more complete understanding of the technic and the interpretation of results of the bacteriological examination of water see *Elements of Water Bacteriology*, by Prescott and Winslow.

For the characteristics of the colon bacilli the Massachusetts State Board of Health uses six media—gelatin, lactose agar, dextrose broth,

milk, nitrate solution, and peptone solution, determining, respectively, absence of liquefaction, production of gas turbidity, coagulation without liquefaction of the coagulum, production of nitrite, and of indol.

Lactose-bile-peptone solution has been much used. In badly contaminated waters this has a distinct advantage in that the bile inhibits many varieties of bacteria more than those of the colon-typhoid group. In good waters the results are very similar from the lactose-peptone and lactose-blue-peptone solutions.

**Significance of the Colon Bacillus.**—The colon test has been received by the majority of engineers and practical sanitarians with great satisfaction, and has been applied with confidence to the examination not only of water, but of shell-fish and other articles of food as well. On the other hand, some have denied its value. Bacteriologists have found bacilli like certain members of the colon group in apparently unpolluted well water. The discovery that animals have colon bacilli identical in the usual characteristics studied with those of man has complicated matters. Thus a fresh hillside stream may be loaded with colon bacilli from the washings of horse or cow manure put on the fields through which it runs or polluted by a stray cow or horse. Swine, hens, birds, etc., may contaminate in unsuspected ways. The number of colon bacilli rather than their presence in any body of surface water is therefore of importance. In well and spring water the presence of colon bacilli indicates contamination. The absence of colon bacilli in water proves its harmlessness so far as bacteriology can prove it. When the colon bacillus is present so as to be isolated from 1 c.c. of water in a series of tests, it is reasonable proof of animal or human pollution and the conditions should be investigated. Ten colon bacilli in 1 c.c. indicates serious pollution. Surface waters from inhabited regions will always contain numerous colon bacilli after a heavy rain, storm, or shower. The washings from roads and cultivated fields contain necessarily large numbers. Winslow reports that in only two out of fifty-eight samples of presumably non-polluted well water did he get colon bacilli in the 1 c.c. samples. Even in twenty-one stagnant pools he only found colon bacilli in five of the 1 c.c. samples.

The experience of all who have studied the subject practically is that in delicacy the colon test surpasses chemical analysis: in constancy and definiteness it also excels the quantitative bacterial count. All these tests must, however, be supplemented by inspection.

**Interpretation of the Quantitative Analysis.**—The older experimenters attempted to establish arbitrary standards by which the sanitary quality of water could be fixed automatically by the number of germs alone. This has been largely given up. Dr. Sternberg considers that a water containing less than 100 bacteria is presumably from a deep source and uncontaminated by surface drainage; that one with 500 bacteria is open to suspicion; and that one with over 1000 bacteria is presumably contaminated by sewage or surface drainage. Even this conservative opinion must be applied with caution. The source of the sample is of vital importance in the interpretation;



thus, a bacterial count which would condemn a spring or well might be normal for a river. In woodland springs and lakes several hundred bacteria per cubic centimeter are frequently found. In lakes the point at which the sample is taken is of great importance, as the bacterial count varies with the distance from the shore and with the depth. The weather also is an influence, since the wind causes currents and waves which stir up the bottom mud, bringing up organisms which have been sedimented. Rains greatly influence streams by flooding them with surface water, bringing a huge number of bacteria at times. The season of the year is an important factor. The counts are highest in the winter and spring months, and lower from April to September.

The following figures illustrate this point:

Water.	Observers.	Year.	Jan.	Feb.	Mar.	April.	May.	June
New York City tap water .	Houghton	1904	890	1100	650	240	350	370
New York City . . . .	Noble	1916	13	17	50	33	6	13
Boston tap water . . . .	Whipple	1892	135	211	102	52	53	86
Merrimac River tap water	Clark	1899	4900	5900	6300	2900	1900	3500

The winter and spring increases are not exceptions to the rule that high numbers indicate danger, but an indication of its truth; for it means a melting of the snow and a flow of surface water into the streams without the usual filtering soil filtration. A number of severe epidemics of typhoid fever have been produced in this way. It is only the fact that typhoid fever is at a minimum in winter that prevents more frequent pollution. Although, as a rule, a series of tests are necessary to pass judgment on a water, a single test may be very important. A large increase in the number in tap water a day after a storm points unerringly to surface pollution, and if towns exist in the water-shed, to street and sewer pollution. The Croton water frequently jumps from hundreds to thousands after such a storm. The low bacterial content in 1916 is due to the addition of chlorine to the water after its leaving the reservoir.

In a typhoid epidemic at Newport, Winslow reports that a test of the water supply showed but 334 bacteria per cubic centimeter, but one from a well showed 6100. The suspicion aroused was justified by finding all the typhoid cases had gotten water from this well.

The study of the bacterial effluent from municipal water filters is the only way in which the efficiency of the filter and the accidents which occur can be determined. In Germany these regular tests are obligatory. The filter should remove about 99 per cent. of the bacteria. Elaborate studies have recently been made of the exact distribution of streams of sewage in bodies of water into which they flow, their disappearance by dilution and sedimentation, and their removal by death. Under peculiar conditions bacteria in water may increase for a time, but here the prevailing bacteria belong almost exclusively to one type.

**Streptococci in Sewage.**—The varieties of streptococci found most often in polluted water correspond to the streptococci described by Houston. In some water in which these are found no *B. coli* have

been found and there is considerable doubt in such cases as to whether the streptococci imply serious pollution. The streptococci remain alive longer than the colon bacilli. In England the examination for streptococci in water is being less regularly done than formerly.

**Other Bacteria.**—Most of the bacteria which develop in the intestines of man and animals necessarily occur in polluted water, and an examination for some of these has been advocated by many, such as the *B. (enteritidis) sporogenes*, other anaërobic spore formers, the various members of the typhoid-colon group, and the proteus group.

**Isolation of the Typhoid Bacillus from Water.**—If it were possible to readily obtain the typhoid bacilli from water, when they were present in small numbers, its examination for that purpose would be of much greater value than it is now; but we have to remember that we can only examine at one time a few cubic centimeters of water by bacteriological methods, and that although the typhoid bacilli may be sufficiently abundant in the water to give, in the quantity that we ordinarily drink, a few bacilli, yet it must be a very lucky chance if they happen to be in the small amount which we examine. Still further, although it is very easy to isolate typhoid bacilli from water when they are in considerable numbers, yet when they are a very minute proportion of all the bacteria present it is almost impossible not to overlook them. Many attempts have been made to devise some method by which the relative number of the typhoid and other parasitic bacteria present in water could be increased at the expense of the saprophytic bacteria. Thus, to 100 c.c. of water 25 c.c. of a 4 per cent. peptone nutrient bouillon is added, and the whole put in the incubator at 38° C. for twenty-four hours. From this, plate cultures are made. As a matter of fact, the typhoid bacillus is rarely found, even in specimens of water where we actually know that it is or has been present because of cases of typhoid fever which have developed from drinking the water. From these facts we must consider our lack of finding the bacillus in any given cases as absolutely no reason for considering the water to be free from danger. Another serious drawback to the value of the examinations for the typhoid bacillus is that they are frequently made at a time when the water is really free from contamination, though both earlier and later the bacillus was present. It is hardly worth while, therefore, except in careful experimental researches, to examine the water for the typhoid bacillus, but rather study the location of the surrounding privies and sources of contamination. A number of observers, resting on the agglutination test, have thought they have isolated typhoid bacilli from the soil and water, but these investigators had not considered sufficiently the matter of group agglutinins, and their results are not trustworthy.

## CONTAMINATION AND PURIFICATION OF DRINKING WATERS.

Brook water and river water are contaminated in two ways: through chemicals, the waste products of manufacturing establishments, and

through harmful bacteria by the contents of drains, sewers, etc., the latter method being far the more dangerous.

When water, which has been soiled by waste products of manufacturing only, becomes so diluted or purified that the contamination is not noticeable to the senses and shows no dangerous products on chemical analysis, it is probably safe to drink. When sewage is the contamination, this rule no longer holds, and there may be no chemical impurities and no pathogenic bacteria found, and yet disease be produced. That river water which has been fouled by sewage will by oxidation, dilution, sedimentation, action of sunlight, and predatory microorganisms, become greatly purified is an indisputable fact. The increase in bacteria which occurs from contamination is also largely or entirely lost after ten to twenty miles of river flow. Nevertheless, the history of many epidemics seems to show that a badly contaminated river is never an absolutely safe water to drink, although with the lapse of each day it becomes less and less dangerous, nor will sand filter-beds absolutely remove all danger. These statements are founded upon the results of numerous investigations; thus the marked disappearance of bacteria is illustrated by the following: Kummel found below the town of Rosbock 48,000 bacteria to the cubic centimeter; twenty-five kilometers farther down the stream only 200 were present—about the same number as before the sewage of Rosbock entered. On the other hand, the doubtful security of depending on a river purification is proved by such experiences as the following: In the city of Lowell, Massachusetts, an alarming epidemic followed the pollution of the Merrimac River three miles above by typhoid feces, and six weeks later an alarming epidemic attacked Lawrence, nine miles below Lowell. It is estimated that the water took ten days to pass from Lowell to Lawrence and through the reservoirs. Typhoid bacilli usually die in river water in from three to ten days, but they may live for twenty-five days in other water; the Lawrence epidemic is easily explained. Newark-on-Trent, England, averaged 75 cases a year from moderately well-filtered water and only 10 when it was changed to deep-well supply.

**Purification of Water on a Large Scale.**—For detailed information on this subject the reader is referred to works on hygiene. Surface waters, if collected and held in sufficiently large lakes or reservoirs, usually become so clarified by sedimentation, except shortly after heavy rains, as to require no further treatment so far as its appearance goes. The collection of water in large reservoirs allows not only the living and dead matter to subside, but allows time also for the pathogenic germs to perish through the influence of light as well as of antagonistic bacteria and other deleterious influences, such as sand, or mechanical coagulant. Filtration of water exerts a very marked purification, taking out 99.8 per cent. of the organisms in those best constructed and at least 95 per cent. in those commonly used in cities. The construction of filters is too large a subject to enter on minutely here; sand filters consist, as a rule, of several layers, beginning with fine sand, and then smaller and larger gravel, and finally rough stones.

A certain time elapses before the best results are obtained; this seems to wait for the formation of a film of organic material on the sand, which is full of nitrifying bacteria. Even the best filters only greatly diminish the dangers of polluted water. Spring water and well water are, in fact, filtered waters.

Water which is subject to serious pollution must be submitted to a preliminary purification before it can be considered a suitable source for a drinking-water supply. The means employed for its purification depend to a large extent upon the character of the water and the nature of the pollution. Filtration through slow sand filters, three to five feet in depth, removes 98 to 99.5 per cent. of the bacteria and organic matter; so that effluents from the best constructed sand filtration beds constitute safe and reliable drinking waters. Five hundred thousand to one or two million gallons, depending somewhat upon the extent of pollution and the fineness of the sand, can be filtered daily per acre. Only the surface of the sand filter becomes in any way clogged and as thin a layer as can be scraped off is removed one or more times a month. This surface sand is washed with clean water and several scrapings replaced at one time. Sand filtration beds are very widely used abroad and are coming into extensive use in this country. The filter-beds at Lawrence, Mass., have been used over ten years with marked success; when properly managed, they render the highly polluted Merrimac River a fairly safe drinking water; the filter-beds there are scraped about thirteen times a year.

Mechanical filtration plants find considerable favor where clarification as well as bacterial purification is desired. A coagulant such as sulphate of aluminum is employed and forms in the water a flocculent precipitate which carries down with it all suspended matter; 125,000,000 or more gallons of water can be filtered on an acre daily, but the filters must be washed daily by reversing the flow and cleansing the clogged filter with a stream of the purified water. Chlorinated lime when added to drinking water to the extent of one-eighth to one-twelfth of a grain per gallon will destroy all intestinal bacteria of the typhoid-colon group within a few hours. This is a very useful means of purification. It does not injure the water and is being used very extensively.

Under special conditions other methods, such as the passage of ozone, have proved successful.

**Domestic Purification.**—Water which requires private filtering should not be supplied for drinking purposes. Unhappily, however, it often is. Domestic filters may be divided, roughly, into those for high and low pressure. The former are directly connected with the water main, while the others simply have the slight pressure of the column of water standing in the filter. Many household filters contain animal charcoal, silicated carbon, etc., either in a pressed condition or in one porous mass. These filters remove much of the deleterious matter from the suspected waters, but the majority cannot be depended upon to remove all bacteria. Even those which are equipped for self-cleansing become foul in a little while, and, if not cleaned, unfit for use. The best of the

filters are of porous stone, such as the Poulton-Berkefeld and Pasteur filters. These yield a water, if too great a pressure is not used, almost absolutely free from bacteria, and if they are frequently cleansed they are reliable. A large Berkefeld filter will allow sixty gallons of water to pass per hour. The Pasteur filter is more compact and slower. From the best Pasteur filters sterile water may be passed for two or three weeks; from the Berkefeld usually only a few days. A single typical low-pressure filter is that of Bailey Denton. The upper compartment contains the filtering material, which may be sand or charcoal, and is fed from a cistern or hydrant. After a certain quantity of water has passed in, the supply is automatically cut off until the whole amount is filtered. A fairly efficient filter is the following: Take a large-sized earthenware pot and plug the hole in the bottom with a cork, through which pass a short glass tube. Upon the bottom place an inch of small pieces of broken flower-pot; upon this a couple of inches of well-washed small gravel, and upon this six to twelve inches of well-washed fine, sharp sand. Cover the sand with a piece of filter-paper and hold this down with a few small stones. Mount the pot on a tripod, and it is ready for use. The paper prevents the sand being disturbed when water is added, and as it also holds most of the sediment, this can be readily removed. Every few months the sand can be washed and replaced. Animal charcoal is not a good substance for permanent filters, as bacteria grow well in it. Whenever water is suspected, and there is any doubt as to the filters, it should be boiled for ten minutes; this will destroy all bacteria. This precaution should always be taken in the presence of typhoid fever and cholera epidemics.

### THE DISPOSAL OF SEWAGE.

The disposal of sewage is becoming a vital question with all towns and cities which are not situated near salt-water outlets, since the present tendency in legislation is to compel such towns to dispose of their waste so that it shall not be a menace to drinking-water streams, destructive to fisheries, or a nuisance to harbors.

Methods of sewage purification depend upon the character of the sewage and the kind of effluent desired.

Two hundred thousand gallons of crude sewage may be filtered upon an acre of land daily and an effluent obtained which will compare favorably in every way known to the chemist and bacteriologist with the best mountain springs. This is, however, a slow process, and it is rare that such a pure effluent is required. Similar results may be obtained by utilizing the septic-tank method, running the sewage from the septic tank to contact beds and thence to sand filter-beds; where, because of the partial "self-purification of the sewage" in the septic tank and contact beds, 2,500,000 gallons of sewage can be filtered daily on an acre of surface. In this process less land is required and both these effluents can be safely turned into drinking-water streams.

If, however, a merely non-putrescible effluent is required, one which,

though high bacterially, will not be offensive in any way, or subject to further decomposition, it may be obtained by passing crude sewage to septic tanks, thence to double contact beds, the resulting effluent having merely an earthy, humus-like odor and being non-putrescible.

Where acid wastes, tannery wastes, dyestuffs, etc., from various factories enter into sewage, its disposal becomes a more complicated problem and chemical precipitation by the use of lime or other chemicals is generally employed for such sewage purification, which at best is only partial and is sometimes supplemented by sand filtration.

**Sea Water.**—This is only feebly bactericidal. The salty tidal waters of rivers allow typhoid bacilli and other members of the typhoid-colon group to live for a number of days.

### BACTERIA IN AIR—EXAMINATION OF AIR.

Saprophytic bacteria are always present in considerable numbers in the air except far out at sea or on high mountains. They reach the air from the earth's surface and are most plentiful nearest to it. They are more abundant where organic matter abounds and in dry and windy weather.

The air is kept constantly in motion by winds so that fine particles are constantly being carried into it from the ground, especially in an inhabited area with its dusty streets. The rays of sunlight visibly reveal these particles to us. The bacteria in the dust of the fields and streets are carried along with these dust particles. They are usually the harmless soil bacteria or the almost equally harmless intestinal bacteria of animals. After a storm few bacteria are in the air, while on a dry windy day many thousands exist in a cubic meter. In warm weather rain carries down the bacteria of the air. The number of bacteria in the air of the country are much less than in the city air. Forests decrease the number of bacteria.

On high mountains and on the sea far from land, bacteria are very scarce. The bacteria that multiply in the soil of street and country are almost entirely saprophytic types. Sunlight and drying rapidly destroy bacteria. In dwellings the bacterial content depends on many factors, of which the chief are the opening of windows to the outside dust-laden air, the cleanliness of the dwelling, and the amount of stirring up of the dust by sweeping. It is almost impossible to separate the effect of the bacteria which we inhale from that of the dust particles which they accompany. Both probably act as slight irritants and so predispose to definite infections.

Except in the air in direct contact with men and animals pathogenic bacteria are present in the air only under exceptional circumstances and usually as spores, such as those of anthrax bacilli from the dust from the wool and hides of infected animals or of tetanus bacilli from the infected manure. The practical results obtained from the examination of air for pathogenic bacteria have been slight. We know that at times they must be in the air, but unless we purposely increase

their numbers they are so few in the comparatively small amount of air which it is practicable to examine that we rarely find them. It is established that in loud speaking, in coughing and sneezing many bacteria from the larynx, fauces and lips are expelled. Examination of dust in hospital wards and sick rooms, in places where only air infection was possible, has occasionally revealed tubercle bacilli and other pathogenic bacteria. Although very light they generally settle to the ground. It is now thought that the factor of air infection in the spread of infectious diseases is of very little importance, except when recent infection has occurred. With the tiny droplets of mucus most of these bacteria die quickly and do not disseminate, while alive, far from the place of their origin.

The simplest method of searching for the varieties of bacteria in the air and their number in any place is to expose to the air for longer or shorter periods nutrient agar spread upon the surface of the Petri dish. After exposure the plates are either put in the incubator at 37° C., or kept at room temperature. When it is desired to obtain the pathogenic bacteria a little rabbit's blood is smeared over the agar. The more careful quantitative examination is made by drawing a given quantity of air through tubes containing sterile sand, which is kept in place by pieces of metal gauze. When the operation is completed the sand is poured into a tube containing melted nutrient gelatin or nutrient agar, and after thorough shaking, the mixture is poured into a Petri dish and the bacteria allowed to develop, either at 37° or 23° C., according as the growth of the parasitic or saprophytic varieties is desired. Instead of agar or gelatin, ascitic broth or animals may be inoculated. Such examinations are occasionally made of the air of theatres, crowded streets in cities, etc. They give the number of non-pathogenic bacteria only.

#### BACTERIOLOGICAL EXAMINATION OF THE SOIL.

The subject from its agricultural side is considered in Chapter LI. Specimens of deep soil can be gathered in sterile, sharp-pointed, sheet-iron tubes. Through the examination, we wish to learn either the number of bacteria or the important varieties of bacteria present. To estimate the number, small fractions of a gram are taken and planted in nutrient agar or in special media contained in Petri dishes. Anaërobic as well as aërobic cultures should be made.

According to Houston, uncultivated sand soil averages 100,000 bacteria per gram, garden soil 1,500,000, and sewage-polluted 115,000,000. The most important bacteria to be sought for are bacilli of the colon group and streptococci. Both of these suggest fairly recent excremental pollution. Tetanus spores may also be found.

The period during which typhoid bacilli remain alive in soil is variable, since it depends on so many unknown factors and differs so in different places. The typhoid bacilli probably rarely increase in the soil and probably rarely survive a month in it. The main danger of soil bacteria is their being washed into water supplied by rains or carried to them by the wind.

## CHAPTER XLIX.

### THE BACTERIOLOGY OF MILK IN ITS RELATION TO DISEASE.

FROM the stand-point of the dairy many of the different varieties of bacteria found in milk are of importance which have little or no medical interest. We have space here only to consider the bacteriology of milk so far as it is related to health and disease. The fermented milks were considered in Chapter XXI. The saprophytic bacteria taken collectively have importance because one can determine from their number something as to the care taken in handling the milk and also because, when numerous, they produce chemical changes in the milk which are harmful for infants. The bacilli of the colon group are of little more importance than any of the saprophytic types, as in the case of milk they simply indicate pollution from the cow which is no more or less harmless than other forms.

**Numerical Estimation of Bacteria.**—The number of bacteria in a cubic centimeter of milk is usually estimated from the colonies developing in nutrient agar plate cultures during a period of forty-eight hours when kept at a temperature of  $37^{\circ}$  C. This allows in market milk in which bacteria have developed at low temperature only a certain proportion of the varieties of bacteria to develop colonies. Sometimes fully twice as many colonies develop at  $20^{\circ}$  to  $27^{\circ}$  C. during three to five days as at  $37^{\circ}$  C. for two days. The advantages of the shorter time and of uniformity have led to the adoption of the technic given in Part I. Any culture method necessarily underestimates their number, as many of the bacteria remain after vigorous shaking in pairs or small groups. In order to overcome this and also to note the morphological types, the direct microscopic examination of smears of the sediment has been urged. A great practical objection to this is that, if a heated milk is examined, the dead as well as the living bacteria are counted, for no satisfactory method has yet been found by means of stains to differentiate between them. It is true that many varieties of bacteria stain less intensely after heating but others do not. This method has, however, great advantages at the creamery or farm in that one can immediately tell whether a sample has few or many bacteria and also note the presence of streptococci and leukocytes. The microscopic count gives from two to ten times as many bacteria as the routine culture method when the individual bacteria are counted. When clumps of bacteria are given the same value as single bacteria the microscopic counts agree closely with the colony counts.

**Smear Method for Direct Examination of Milk.**—The Prescott-Breed method is the most accurate. That of Slack is also used. (See below.)



**Advantages and Disadvantages of the Direct or Microscopic Method of Milk Analysis.**—**Advantages.**—1. The counting of each organism seen, while difficult and time-consuming, makes it possible to ascertain approximately the actual number of bacteria in milk. The counting of the clumps as one organism approximates the colony count, but loses the value of an exact estimation.

2. It eliminates the greater portion of glassware required at present in the plate method, the preparation of agar, etc., which lessens the initial cost.

These advantages, however, accrue mainly to the dealers employing a bacteriologist to make the milk analyses at the source.

**Disadvantages.**—The disadvantages of the direct method are: 1. The very small quantity of milk used for counting leads to inaccuracy.

2. That as a resultant of the foregoing facts large factors have to be used in estimating the bacterial content of milk, with the consequent introduction of large factors of error.

3. In estimating the bacterial content at all accurately of a very good milk, large numbers of fields have to be examined with a consequent expenditure of time, which increases the cost.

4. The individuality of the counter enters more largely into this method than in the plate method, and a better trained bacteriologist is necessary.

Samples graded on colony count, bacterial content per c.c.	No. of samples.	Average plate count.	Average microscopic count of individual organisms.	Ratio.
Less than 50,000 . . . . .	594	14,491	92,034	1: 6.29
50,000 to 300,000 . . . . .	133	103,075	708,681	1: 6.87
300,000 to 1,500,000 . . . . .	42	648,452	5,254,018	1: 8.1
1,500,000 to 5,000,000 . . . . .	13	2,538,461	21,541,534	1: 8.48
5,000,000 or over . . . . .	8	8,575,000	27,519,531	1: 4.18
Total . . . . .	790	171,181	1,100,997	1: 6.43

Prescott and Breed explain their smear method briefly as follows: The sample of milk to be examined is shaken thoroughly and 0.01 c.c. is withdrawn by means of a specially constructed capillary pipette. The milk so obtained is spread evenly over an area of one square centimeter on an ordinary glass slide. These areas may be easily determined by placing the glass slide over paper or glass on which areas of this size have been accurately ruled out. More satisfactory results can be obtained by using circular instead of square areas. Duplicate smears should be made on the same slide. The milk is then dried with gentle heat, the fat dissolved out with xylol or other fat solvent, the smear again dried, immersed in alcohol for a few minutes to fix the film, again dried and stained with methylene blue or other stain. Alkaline stains or others which attack the casein and loosen the smear must be avoided. The glassware used must be cleansed but need not be sterilized, as the bacteria have no chance to increase in number.

The counting of the bacteria is done by a microscope and an oil-immersion lens. If the diameter of the field is so adjusted, by means of the draw tube, that it equals 0.16 mm., then each field of the micro-

scope covers approximately one-five-thousandth (0.0002) of a square centimeter. On this basis each bacterium seen in a field taken at random represents 500,000 per c.c. if they are evenly distributed. However, it is impossible to distribute them evenly so that at least 110 fields of the microscope should be counted when there are many bacteria, 50 fields when the number is moderate and 100 fields if accurate results are required. The total number of bacteria seen in 10 fields multiplied by 50,000 or the total number seen in 100 fields multiplied by 5000 gives approximately the total number of bacteria per cubic centimeter. The counts vary considerably when individual bacteria are counted, even when made by the same examiner, because of one or more large clumps being in one series of fields and not in another. Even when clumps are counted as one the distribution varies in the different fields.

COMPARISON BETWEEN MICROSCOPIC COUNTS BY DIFFERENT EXAMINERS.

Examiner.	
A . . . . .	Count of individual bacteria 164,000
	Count of clumps . . . . . 65,000
B . . . . .	Count of individual bacteria 29,000
	Count of clumps . . . . . 8,000
C . . . . .	Count of individual bacteria 133,000
	Count of clumps . . . . . 38,000

**Varieties.**—Bacteria in milk can be divided into two great groups—those which get into the milk after it leaves the udder and those which come from the cow. The first group comprises bacteria from dust, hands, milking pails, strainers, etc.

The extraneous bacteria are of importance because they indicate the conditions under which the milk was produced and cared for and because they produce changes in the chemical composition of the milk when they have developed in great numbers. The number of bacteria in any sample of milk depends on three factors: the number deposited in the milk from the cow's udder, from the air, and utensils; the time during which they have developed, and the temperature at which the milk has stood. The last is perhaps the most important factor. The attempt was made during a period of one year to connect illness in infants and children with special varieties of saprophytic bacteria in milk, but with negative results.

From the milks altogether 239 varieties of bacteria were isolated and studied. These 239 varieties, having some cultural or other differences, were divided into 31 classes, each class containing from 1 to 39 more or less related organisms.

As to the sources of bacteria found in milk, we made sufficient experiments to satisfy us that they came chiefly from outside the udder and milk ducts.

Bacteria were isolated from various materials which under certain conditions might be sources of contamination for the milk, and the cultures compared with those taken from milk. Thus there were obtained from 20 specimens of hay and grass, 31 varieties of bacteria;

from 15 specimens of feces, manure, and intestinal contents, 28 varieties; from 10 specimens of feed, 17 varieties. Of these 76 varieties there were 42 which resembled closely those from milk—viz., 11 from grass or hay; 26 from manure; 5 from feed.

During the investigation a number of the varieties isolated from milk were shown to be identical with types commonly found in water.

From the few facts quoted above and from many other observations made during the course of the work it would seem that the term "milk bacteria" assumes a condition which does not exist in fact. The expression would seem to indicate that a few varieties, especially those derived in some way from the cow, are commonly found in milk, which forms having entered the milk while still in the udder or after its withdrawal, are so well fitted to develop in milk that they outgrow all other varieties.

As a matter of fact it was found that milk taken from a number of cows, in which almost no outside contamination had occurred, and plated immediately, contained, as a rule, very few bacteria, and these were streptococci, staphylococci, and other varieties of bacteria not often found in milk sold in New York City; the temperature at which milk is kept being less suitable for them than for the bacteria which fall into the milk from dust, manure, etc. A number of specimens of fairly fresh market milk averaging 200,000 bacteria per cubic centimeter were examined immediately, and again after twelve to twenty-four hours. In almost every test the three or four predominant varieties of the fresher milk remained as the predominant varieties after the period mentioned.

The above experiments seem to show that organisms which have gained a good percentage in the ordinary commercial milk at time of sale will be likely to hold the same relative place for as long a period as milk is usually kept. After the bacteria pass the ten or twenty million counts a change occurs, since the increasing acidity inhibits the growth of some forms before it does that of others. Thus some varieties of the lactic acid bacteria can increase until the acidity is twice as great as that which inhibits the growth of many bacteria. Before milk reaches the curdling point, the bacteria may have reached over a billion to each cubic centimeter. For the most part specimens of milk from different localities showed a difference in the character of the bacteria present, in the same way that the bacteria from hay, feed, etc., varied. Even the intestinal contents of cows, the bacteriology of which might be expected to show common characteristics, contained, besides the predominating colon types, other organisms, which differed widely in different species and in different localities. Cleanliness in handling the milk and the temperature at which it had been kept were also found to have a marked influence on the predominant varieties of bacteria present.

**Pathogenic Properties of the Bacteria Isolated.**—Intraperitoneal injection of 2 c.c. of broth or milk cultures of about 40 per cent. of the varieties tested caused death. Cultures of most of the remainder

produced no apparent deleterious effects even when injected in larger amounts. The filtrates of broth cultures of a number of varieties were tested, but only one was obtained in which poisonous products were abundantly present. Death in guinea-pigs weighing 300 grams followed within fifteen minutes after an injection of 2 c.c.; 1 c.c. had little effect.

As bacteria in milk are swallowed and not injected under the skin, it seemed wise to test the effect of feeding them to very young animals. We therefore fed forty-eight cultures of 139 varieties of bacteria to kittens of two to ten days of age by means of a glass tube. The kittens received 5 to 10 c.c. daily for from three to seven days. Only one culture produced illness or death. Very young guinea-pigs were fed in the same manner with similar results.

After five years of effort to discover some relation between special varieties of bacteria found in milk and the health of children the conclusion has been reached that neither through animal tests nor the isolation from milk given sick infants have we been able to establish such a relation. Pasteurized or "sterilized" milk is rarely kept longer than thirty-six hours, so that varieties of bacteria which after long standing develop in such milk did not enter into our problem. The harmlessness of cultures given to healthy young kittens does not, of course, prove that they would be equally harmless in infants. Even if harmless in robust infants, they might be injurious when summer heat and previous disease had lowered the resistance and the digestive power of the subjects.

**Streptococci in Relation to Disease.**—In an investigation by Dr. D. H. Bergey connection between diarrhea and pus and streptococci was sometimes found.

The results of this investigation appear to warrant the following conclusions:

1. The occurrence of an excessive number of leukocytes in cows' milk is probably always associated with the presence in the udder of some inflammatory reaction brought about by the presence of some of the ordinary pyogenic bacteria, especially of streptococci.

2. When a cow's udder has once become infected with the pyogenic bacteria, the disease tends to persist for a long time, probably extending over several periods of lactation.

3. Lactation has no causative influence *per se* upon the cellular and bacterial content of cows' milk, though it probably tends toward the aggravation of the disease when the udder is once infected.

It is impossible to differentiate in routine milk examinations the pathogenic streptococci of diseased cows from saprophytic varieties. Thus it happens that a milk which contains great numbers of streptococci may or may not be more dangerous than one which contains an equal number of other apparently less harmful bacteria. The identification of the varieties present requires great care and is only done in the face of feared infection such as an epidemic of septic throats. Those that produce human diseases, except in infants, are probably always from cows in which the udder has been infected from human sources.

**The Deleterious Effect of Live Bacteria in Milk on Infants.**—We have tested this ourselves in the following way: During each of the summers of 1902, 1903, and 1904 a special lot of milk was modified for a group of fifty infants, all of whom were under nine months of age, and distributed daily. To one half a portion of the milk was given raw; to the other half a portion heated at 60° for twenty minutes.

The modified milk was made from a fairly pure milk mixed with ordinary cream. The bacteria contained in the milk numbered on the average 45,000 per cubic centimeter, in the cream 30,000,000. The modified raw milk taken from the bottles in the morning averaged 1,200,000 bacteria per cubic centimeter, or considerably less than the ordinary grocery milk; the pasteurized, about 1000; taken in the late afternoon of the same day they had, respectively, about 20,000,000 and 50,000.

Twenty-one predominant varieties of bacteria were isolated from six specimens of this milk collected on different days. The varieties represented the types of bacteria frequently found in milk. The infants were selected during the first week in June, and at first all were placed on pasteurized milk. The fifty infants which had been selected were now separated into two groups as nearly alike as possible. On the fifteenth of June the milk was distributed without heating to one half the infants, the other half receiving as before the heated milk. In this way the infants in the two groups received milk of identically the same quality, except for the changes produced by heating to 165° F. for thirty minutes. The infants were observed carefully for three months and medical advice was given when necessary. When severe diarrhea occurred barley water was substituted for milk.

The first season's trial gave the following results: Within one week 20 out of 27 infants put on raw milk suffered from moderate or severe diarrhea; while during the same time only 5 cases of moderate and none of severe diarrhea occurred in those taking pasteurized milk. Within a month 8 of the 27 had to be changed from raw back to heated milk, because of their continued illness; 7, or 25 per cent., did well all summer on raw milk. On the other hand, of those receiving the pasteurized milk, 75 per cent. remained well, or nearly so, all summer, while 25 per cent. had one or more attacks of severe diarrhea. There were no deaths in either group of cases.

During the second summer a similar test was made with 45 infants. Twenty-four were put on raw modified milk; 13 of these had serious diarrhea, in 5 of whom it was so severe that they were put back upon heated milk; 10 took raw milk all summer without bad effects; 2 died, 1 from gross neglect on the part of the mother, the other from diarrhea. Of the 21 on pasteurized milk, 5 had severe attacks of diarrhea, but all were kept on this milk except for short periods, when all food was omitted; 16 did well throughout the summer. One infant, markedly rachitic, died. The third summer's results have not been tabulated, but were similar to those of the first two tests.

The outcome of the these observations during the first two summers are summarized in the following table:

Kinds of milk. Number of bacteria when consumed.	Number of infants.	Remained well for entire summer.	Number having severe or moderate diarrhea.	Average number days off milk during summer.	Average weekly gain in weight, oz.	Average number of days diarrhea.	Deaths.
Pasteurized milk, 1000-50,000 bacteria per c.c.	41	31	10	3.0	4.0	3.9	1
Raw milk, 1,200,000-20,000,000 bacteria per c.c.	51 <sup>1</sup>	17	33	5.5	3.5	11.5	2

Although the number of cases was not large, the results, almost identical during the three summers, indicate that even a fairly pure milk, when given raw in hot weather, causes illness in a much larger percentage of cases than the same milk given after pasteurization. A considerable percentage of infants, however, do apparently quite as well on raw as on pasteurized milk.

**Bacteria in Milk. Effect on Older Children.**—The children over three years of age who received unheated milk, containing at different times from 145,000 to 350,000,000 bacteria per cubic centimeter, showed almost no gastro-intestinal disturbance. The conditions at three institutions will serve as examples.

In the first of these an average grade of raw milk was used which, during the summer, contained from 2,000,000 to 30,000,000 bacteria per cubic centimeter. This milk was stored in an ice-box until required. It was taken by children unheated and yet no case of diarrhea of sufficient gravity to send for a physician occurred during the entire summer. This institution was an orphan asylum containing 650 children from three to fourteen years of age—viz., three to five years, 98; five to eight years, 162; eight to fourteen years, 390.

A second institution used an unheated but very pure milk which was obtained from its own farm. This milk averaged 50,000 bacteria per cubic centimeter. The inmates were 70 children of ages ranging from three to fourteen years. In this institution not a single case of diarrheal disease of any importance occurred during the summer.

In a third institution an average grade of milk was used which was heated. This milk before heating contained 2,000,000 to 20,000,000 bacteria per cubic centimeter. This institution was an infant asylum in which there were 126 children between the ages of two and five years. There were no cases of diarrhea during the summer.

These clinical observations taken in connection with the bacteriological examination at the laboratory show that although the milk may come from healthy cattle and clean farms and be kept at a tem-

<sup>1</sup> Thirteen of the fifty-one infants on raw milk were transferred before the end of the trial to pasteurized milk because of serious illness. If these infants had been left on raw milk it is believed by the writers that the comparative results would have been even more unfavorable to raw milk.

perature not exceeding 60° F., a very great increase in the number of bacteria may occur. Furthermore, this may occur without the accumulation in the milk of sufficient poisonous products or living bacteria to cause appreciable injury in children over three years of age, even when such milk is consumed in considerable amount and for a period extending over several months. Milk kept at temperatures somewhat above 60° F. was not met with in our investigations, but the histories of epidemics of ptomain poisoning teach that such milk may be very poisonous. It is also to be remembered that milk abounding in bacteria on account of its being carelessly handled is also always liable to contain pathogenic organisms derived from human or animal sources.

**Results with Very Impure Milk Heated vs. Those with Pure or Average Milk Heated.**—During the summer of 1901 we were able to observe a number of babies fed on milk grossly contaminated by bacteria. In 1902 systematic supervision of all stores selling milk was instituted by the Health Department, so that the very worst milk was not offered for sale that summer.

The observations upon the impure milk of 1901 are of sufficient importance to be given in detail, although already mentioned in the report of the observations upon infants of both summers which were fed on "store milk." A group of over 150 infants was so divided that 20 per cent. were allowed to remain on the cheapest store milk which they were taking at the time. To about the same number was given a pure bottled milk. A third group was fed on the same quality of milk as the second, but sterilized and modified at the Good Samaritan Dispensary. A fourth group received milk from an ordinary dairy farm. This milk was sent to a store in cans and called for by the people. A few infants fed on breast and condensed milk were observed for control.

In estimating the significance of the observations recorded in the tables, one should bear in mind that not only do different infants possess different degrees of resistance to disease, but that, try as hard as the physicians could, it was impossible to divide the infants into groups which secured equal care and were subjected to exactly the same conditions. It was necessary to have the different groups in somewhat different parts of the city. It thus happened that the infants on the cheap store milk received less home care than the average, and that those on the pure bottled milk lived in the coolest portion of the city. Certain results were, however, so striking that their interpretation is fairly clear. It is to be noted that the number of infants included in each group is small.

There is nothing in the observations to show that fairly fresh milk from healthy cows, living under good hygienic conditions and containing, on some days, when delivered, as many as 200,000 bacteria per cubic centimeter, had any bacteria or any products due to bacteria that remained deleterious after the milk was heated to near the boiling-point.

# RESULTS WITH IMPURE AND PURE MILK HEATED 631

TABLE SHOWING THE RESULTS OF FEEDING DURING JULY AND AUGUST, 1901, IN TENEMENT HOUSES, OF 112 BOTTLE-FED INFANTS UNDER ONE YEAR OF AGE, AND OF 47 BOTTLE-FED INFANTS BETWEEN ONE AND TWO YEARS OF AGE WITH MILK FROM DIFFERENT SOURCES, AND THE NUMBER OF BACTERIA PRESENT IN THE MILK.

Character of milk.	Infants under one year.					Infants over one year.				
	Number of infants.	Average weekly gain.	Diarrhea.		Deaths.	Number of infants.	Average weekly gain.	Diarrhea.		Deaths.
			Mild.	Severe.				Mild.	Severe.	
1. Pure milk boiled and modified at dispensary or stations; given out in small bottles. Milk before boiling averaged 20,000 bacteria per c.c.; after boiling 2 per c.c.	41	3 oz.	10	8	1 <sup>1</sup>					
2. Pure milk, 24 hours old, sent in in quart bottles to tenements, heated and modified at home, 20,000 to 200,000 bacteria per c.c. when delivered.	23	4½ oz.	8	5	0	24	4½ oz.	8	2	0
3. Ordinary milk, 36 hours old, from a selected group of farms, kept cool in cans during transport; 1,000,000 to 25,000,000 bacteria per c.c., heated and modified at home before using.	18	4 oz.	6	6	1 <sup>1</sup>	12	4 oz.	1	2	0
4. Cheap milk, 36 to 60 hours old, from various small stores, derived from various farms, some fairly clean, some very dirty; 400,000 to 175,000,000 bacteria per c.c. before home heating.	21	½ oz.	4	13	4 <sup>2</sup>	7	½ oz.	1	3	0
5. Condensed milk of different brands. Made up with hot water. As given, contained bacteria from 5,000 to 200,000 per c.c.	9	½ oz.	5	2	3	4	3½ oz.	1	3	0
6. Breast milk . . . . .	16	2½ oz.	5	2	0					

On the other hand, it is possible that certain varieties of bacteria may, under conditions that are insanitary, find entrance to milk and survive moderate heat or may develop poisonous products resistant to heat in sufficient amount to be harmful, even when they have accumulated to less than 200,000 per c.c.

Turning now to the results of feeding with milk which has been heated and which before sterilization contained from 1,000,000 to 25,000,000 bacteria per cubic centimeter, averaging about 15,000,000, though obtained from healthy cows living under fairly decent conditions and although the milk was kept moderately cool in transit, we find a distinct increase in the amount of diarrheal diseases. Though it is probable that the excessive amount of diarrhea in this group of children was due to bacterial changes which were not neutralized by heat or to living bacteria which were not killed, yet it is only fair to consider that the difference was not very great and that the infants of

<sup>1</sup> This infant died from enteritis and toxemia.

<sup>2</sup> This infant died of pneumonia. There had been no severe intestinal disorder noted.

<sup>3</sup> One of the four had pertussis, the remaining three died from uncomplicated enteritis.



this group were under surroundings not quite so good as those on the pure milk.

Finally, we come in this comparison to the infants who received the cheap store milk after heating. This milk had frequently to be returned because it curdled when boiled, and contained, according to the weather, from 4,000,000 to 200,000,000 bacteria per cubic centimeter. In these infants the worst results were seen. This is shown not only by the death-rate, but by the amount and by the severity of the diarrheal diseases, and the general appearance of the children as noted by the physicians. Although the average number of bacteria in the milk received by this group is higher than that received by the previous group, the difference in results between this group and the previous one can hardly be explained by the difference in the number of bacteria. The varieties of bacteria found in this milk were more numerous than in the better milk, but we were unable to prove that they were more dangerous. Probably the higher temperature at which the milk was kept in transit, and the longer interval between milking and its use, allowed more toxic bacterial products to accumulate.

**Bacterial Contamination of Milk—General Conclusions<sup>1</sup> as to Relative Importance.**—1. During cool weather neither the mortality nor the health of the infants observed in the investigation was appreciably affected by quality of the market milk or by the number of bacteria which it contained. The different grades of milk varied much less in the amount of bacterial contamination in winter than in summer, the store milk averaging only about 750,000 bacteria per cubic centimeter.

2. During hot weather, when the resistance of the children was lowered, the kind of milk taken influenced both the amount of illness and the mortality; those who took condensed milk and cheap store milk did the worst, and those who received breast milk, pure bottled milk, and modified milk did the best. The effect of bacterial contamination was very marked when the milk was taken without previous heating; but, unless the contamination was very excessive, only slight when heating was employed shortly before feeding.

3. The number of bacteria which may accumulate before milk becomes noticeably harmful to the average infant in summer differs with the nature of the bacteria present, the age of the milk, and the temperature at which it has been kept. When the milk is taken raw, the fewer the bacteria present the better are the results. Of the usual varieties, over 1,000,000 bacteria per cubic centimeter are certainly deleterious to the average infant. However, many infants take milk without apparently harmful results. Heat of 145° F. for thirty minutes or of 170° F. for a shorter period not only destroys most of the bacteria present, but, apparently, some of their poisonous products. No harm from the bacteria previously existing in recently heated

<sup>1</sup> These conclusions were drawn up by the writer in association with Dr. L. E. Holt, after a joint study of the results obtained in the studies above recorded.

milk was noticed in these observations unless they had amounted to many millions, but in such numbers they were decidedly deleterious.

4. When milk of average quality was fed, pasteurized and raw, those infants who received milk previously heated did, on the average, much better in warm weather than those who received it raw. The difference was so quickly manifest and so marked that there could be no mistaking the meaning of the results.

5. No special varieties of bacteria were found in unheated milk which seemed to have any special importance, in relation to the summer diarrheas of children. A few cases of acute indigestion were seen immediately following the use of pasteurized milk more than thirty-six hours old. Samples of such milk were found to contain more than 100,000,000 bacteria per cubic centimeter, mostly spore-bearing varieties. The deleterious effects, though striking, were neither serious nor lasting.

6. After the first twelve months of life infants are less and less affected by the bacteria in milk derived from healthy cattle and the air. According to these observations, when the milk had been kept cool, the bacteria did not appear to injure the children over three years of age at any season of the year, unless in very great excess.

7. While it is true that even in tenements the results with the best bottle feeding are nearly as good as average breast feeding, it is also true that most of the bottle feeding is at present very badly done; so that, as a rule, the immense superiority of breast feeding obtains. This should therefore be encouraged by every means, and not discontinued without good and sufficient reasons. The time and money required for artificial feeding, if expended by the tenement mother to secure better food and more rest for herself, would often enable her to continue nursing with advantage to her child.

**Influence of Temperature upon the Multiplication of Bacteria in Milk.**--Few, even of the well informed, appreciate how great a difference a few degrees of temperature will make in the rate of bacterial multiplication. Milk rapidly and sufficiently cooled keeps almost unaltered for thirty-six hours, while milk insufficiently cooled deteriorates rapidly.

The majority of the bacteria found in milk grow best at temperatures above 70° F., but they also multiply slowly even at 40° F.; thus, of 60 species isolated by us, 42 developed good growth at the end of seven days at 39° F. Our observations have shown that the bacteria slowly increase in numbers after the germicidal properties of the milk have disappeared, and the germs have become accustomed to the low temperature. In fact, milk cannot be permanently preserved unaltered unless kept at 32° F. or less. The degree of cooling to which ordinary supplies of milk are subjected differs greatly in various localities. Some farmers chill their milk rapidly, by means of pipe coils over which the milk flows; others use deep wooden tanks filled with water into which the cans of milk are placed soon after milking. In winter these methods are very satisfactory, for the water runs into the pipes or tanks at about 38° F. In warmer weather they

are unsatisfactory, unless ice is used, as the natural temperature of the water may be as high as 55° F. A considerable quantity of milk is not cooled at all at the farms. It is sent to the creamery or railroad after two to six hours, and is then more or less cooled. These few hours in summer, when the milk is left almost at blood heat, allow an enormous development of bacteria to take place, as is shown in the table below.

TABLE I.—SHOWING THE DEVELOPMENT OF BACTERIA IN TWO SAMPLES OF MILK MAINTAINED AT DIFFERENT TEMPERATURES FOR TWENTY-FOUR, FORTY-EIGHT, AND NINETY-SIX HOURS, RESPECTIVELY. THE FIRST SAMPLE OF MILK WAS OBTAINED UNDER THE BEST CONDITIONS POSSIBLE, THE SECOND IN THE USUAL WAY. WHEN RECEIVED, SPECIMEN, NO. 1 CONTAINED 3000 BACTERIA PER C.C., SPECIMEN NO. 2, 30,000 PER C.C.

Temperature. Fahrenheit.	Time which elapsed before making test.			
	24 hours.	48 hours.	96 hours.	168 hours.
32°	2400 <b>30,000<sup>1</sup></b>	2100 <b>27,000</b>	1850 <b>24,000</b>	1400 <b>19,000</b>
39°	2500 <b>38,000</b>	3600 <b>58,000</b>	218,000 <b>4,300,000</b>	4,209,000 <b>38,000,000</b>
42°	2600 <b>43,000</b>	3600 <b>210,000</b>	500,000 <b>5,760,000</b>	11,200,000 <b>120,000,000</b>
46°	3100 <b>42,000</b>	12,000 <b>360,000</b>	1,480,000 <b>12,200,000</b>	80,000,000 <b>300,000,000</b>
50°	11,600 <b>89,000</b>	540,000 <b>1,940,000</b>	300,000,000 <b>1,000,000,000<sup>2</sup></b>	1,000,000,000 <sup>2</sup>
55°	18,800 <b>187,000</b>	3,400,000 <b>38,000,000</b>		
60°	180,000 <b>900,000</b>	28,000,000 <b>168,000,000</b>		
68°	450,000 <b>4,000,000</b>	500,000,000 <b>1,000,000,000<sup>2</sup></b>		

OBSERVATIONS ON BACTERIAL MULTIPLICATION IN MILK AT 90° F., A TEMPERATURE COMMON IN NEW YORK IN HOT SUMMER WEATHER.

TABLE II.—NUMBER OF BACTERIA PER C.C.

	Milk I. Fresh and of good quality.	Milk II. Fair quality from store.	Milk III. Bad quality from store.
Original number . .	5,200	92,000	2,800,000
After two hours . .	8,400	184,000	4,220,000
After four hours . .	12,400	470,000	19,000,000
After six hours . .	68,500	1,260,000	39,000,000
After eight hours . .	654,000	6,800,000	124,000,000

A sample of milk No. 1 removed after six hours and cooled to 50° F. contained 145,000,000 at the end of twenty-four hours. Some of this milk, kept cool from the beginning contained but 12,800 bacteria per cubic centimeter at the end of twenty-four hours.

**Pasteurization of Milk.**—The two dominant factors which control the temperature and time at which the milk should be heated are (1) the thermal death-points of pathogenic bacteria, and (2) the thermolabile food constituents of the milk. The first factor is almost equally important for milk used by persons of all ages, while the second factor is only important for milk used for very young children.

The exposure of bacteria for a short time at a high temperature

<sup>1</sup> The figures referring to tests of the second sample are printed in heavy-face type.

<sup>2</sup> These figures signify the maximum growth and are conservative estimates only.

is equivalent to a longer time at a lower temperature. The ferments and other labile food constituents, on the other hand, are altered much more by the higher temperature. It is well, therefore, to choose the lowest possible temperature which will kill the non-spore-bearing pathogenic bacteria in a practicable length of time. Such an exposure is 60° C. (140° F.) for thirty minutes, 70° C. (158° F.) for five minutes. Very much shorter exposures, as one minute at 70° C., will kill the great majority of pathogenic and other bacteria in the milk and add much of safety, as seen in the tables below, but it is better to be on the safe side.

TABLE SHOWING EFFECT OF HEAT UPON TUBERCLE BACILLI IN MILK.

Degree of heat.	Time exposed.	Amount of milk.	Result in guinea-pigs.
60° C.	15.0 min.	1 c.c.	Infection
60° C.	20.0 min.	1 c.c.	No infection
60° C.	30.0 min.	1 c.c.	No infection
70° C.	0.5 min.	1 c.c.	Infection <sup>1</sup>
70° C.	1.0 min.	1 c.c.	No infection
70° C.	2.0 min.	1 c.c.	No infection
Control not heated		0.001	Infection

This milk was infected by adding one-fifth of its quantity of sputum rich in tubercle bacilli.

**Development of Bacteria in Milk which has been Heated.**—There is a common idea that bacteria develop much more rapidly in milk that has been heated than in raw milk. This is only true for freshly drawn milk which has slight bactericidal power.

The table below shows the effect on bacteria in milk of heating to 70° C. for one-half and one minute. Not only the immediate reduction in number is seen to be great, but the difference continues when the milk is kept cold for two days.

TWO SAMPLES MIXED FROM 100 SAMPLES FROM INSPECTORS. PASTEURIZED AT 160° F. PLATES MADE SAME DAY.

Sample I.		Sample II.	
Raw milk . . . . .	600,000	Raw milk . . . . .	5,400,000
$\frac{1}{2}$ minute pasteurized . . . . .	2,000	$\frac{1}{2}$ minute pasteurized . . . . .	7,400
1 minute pasteurized . . . . .	1,000	1 minute pasteurized . . . . .	600

SAME SAMPLES KEPT IN ICE-BOX TWENTY-FOUR HOURS AT 45° F. (7° C.).

Raw milk . . . . .	6,300,000	Raw milk . . . . .	21,600,000
$\frac{1}{2}$ minute pasteurized . . . . .	18,000	$\frac{1}{2}$ minute pasteurized . . . . .	12,000
1 minute pasteurized . . . . .	900	1 minute pasteurized . . . . .	3,600

IN ICE-BOX FORTY-EIGHT HOURS AT 45° F. (7° C.).

Raw milk . . . . .	16,200,000	Raw milk . . . . .	63,000,000
$\frac{1}{2}$ minute pasteurized . . . . .	120,000	$\frac{1}{2}$ minute pasteurized . . . . .	276,000
1 minute pasteurized . . . . .	10,000	1 minute pasteurized . . . . .	90,000

IN ROOM AT 71° F. (22° C.).

Raw milk . . . . .	36,600,000	Raw milk . . . . .	150,000,000
$\frac{1}{2}$ minute pasteurized . . . . .	5,460,000	$\frac{1}{2}$ minute pasteurized . . . . .	4,500,000
1 minute pasteurized . . . . .	5,400,000	1 minute pasteurized . . . . .	3,600,000

<sup>1</sup> Most of the guinea-pigs were not infected by the milk heated for one-half minute.

## NUMBER OF BACTERIA IN MILK PRODUCED UNDER DIFFERENT CONDITIONS.

1. The number of bacteria present at the time of milking and twenty-four, forty-eight, and seventy-two hours afterward in milk obtained and kept under correct conditions.

No preservatives were present in any of the following specimens:

Pure milk obtained where every reasonable means was taken to ensure cleanliness. The long hairs on the udder were clipped; the cows roughly cleaned and placed in clean barns before milking; the udders were wiped off just previous to milking; the hands of the men were washed and dried; the pails used had small (six-inch) openings, and were thoroughly cleaned and sterilized by steam before use. Milk cooled within one hour after milking to 45° F., and subsequently kept at that temperature. The first six specimens were obtained from individual cows; the last six from mixed milk as it flowed at different times from the cooler. Temperature of barns 55° F.

## NUMBER OF BACTERIA IN 1 C.C. OF MILK.

FROM SIX INDIVIDUAL COWS.			
5 hours after milking.	After 24 hours.	After 48 hours.	After 72 hours.
500	700	12,500	Not counted.
700	700	29,400	Not counted.
19,900	5200	24,200	Not counted.
400	200	8600	Not counted.
900	1600	12,700	Not counted.
13,600	3200	19,500	Not counted.
Average 6,000	1933	17,816	

## FROM SAMPLES OF MIXED MILK OF ENTIRE HERD.

6900	12,000	19,800	494,000
6100	2200	20,200	550,000
4100	700	7900	361,000
1200	400	7100	355,000
6000	900	9800	445,000
1700	400	8700	389,000
Average 4333	2766	10,583	329,000

Twenty-five samples taken separately from individual cows on another day and tested immediately averaged 4550 bacteria per cubic centimeter and 4500 after twenty-four hours. These twenty-five specimens were kept at between 45° and 50° F.

2. Milk taken during winter in well-ventilated, fairly clean, but dusty barns. Visible dirt was cleaned off the hair about the udder before milking. Milkers' hands were wiped off, but not washed. Milk pails and cans were clean, but the straining cloths dusty. Milk cooled within two hours after milking to 45° F.

## NUMBER OF BACTERIA IN 1 C.C. OF MILK.

At time of milking.	After 24 hours.	After 48 hours.
12,000	14,000	57,000
13,000	20,000	65,000
21,500	31,000	106,000
Average 15,500	21,666	76,000

## NUMBER IN CITY MILK.

3. The condition of the average raw city milk is very different, and is shown in the following tables.

The twelve samples were taken late in March, 1912, by Inspectors of the Department of Health of New York City from cans of milk immediately upon their arrival in the city. Raw milk at present gives similar counts.

The temperature of the atmosphere averaged 50° F. during the previous twenty-four hours. The temperature of the milk when taken from the cans averaged 45° F. Much of this milk had been carried over two hundred miles. From the time of its removal from the cans, which was about 2 A.M., until its distribution in nutrient agar, at 10 A.M., the milk was kept at about 45° F.

## FROM NEW YORK AND HUDSON RIVER RAILROAD.

## FROM HARLEM RAILROAD.

No. of sample.	No. of bacteria in 1 c.c.	No. of sample.	No. of bacteria in 1 c.c.
50 . . . . .	35,200,000	48 . . . . .	6,200,000
51 . . . . .	13,000,000	49 . . . . .	2,200,000
52 . . . . .	2,500,000	50 . . . . .	15,000,000
53 . . . . .	1,400,000	51 . . . . .	70,000
54 . . . . .	200,000	52 . . . . .	80,000
55 . . . . .	600,000	53 . . . . .	320,000

The results of the examination of many thousands of specimens last year indicated that most of the milk of grade A was of fair quality, but that the raw milk of the other grades contained excessive numbers of bacteria before pasteurization.

It must be kept in mind that milk averaging 3,000,000 bacteria per cubic centimeter will, when kept at the temperature common in the homes of the poor who comprise the larger part of the population, soon contain very largely increased numbers and show its dangerous condition by turning sour and curdling.

**Cleanliness Used in Obtaining Milk, and Its Influence.**—The present conditions under which much of the milk is obtained are not pleasant to consider. In winter, and to a less extent at other seasons of the year, the cows in many stables stand or lie down in stalls in the rear portion of which there is altogether too much manure and urine. When milked the hands of the milkers are not cleansed, nor are the under portions of the cows, only visible masses of manure adhering to the hair about the udder being removed. Some milkers even moisten their hands with milk, to lessen friction, and thus wash off the dirt of their hands and the cow's teats into the milk in the pails. Some may regard it as an unnecessary refinement to ask that farmers should thoroughly clean the floors of their stalls once each day, that no sweeping should be done just before milking, and that the udders should be wiped with a clean damp cloth and the milkers should thoroughly wash and wipe their hands before commencing milking. The pails and cans should not only be carefully cleansed, but afterward scalded out with boiling water. The washing of the hands would lessen the number of ordinary filth bacteria in the milk, and diminish risk of transmitting to milk human infectious diseases, like scarlet fever, diphtheria, and enteric fever, by the direct washing off of the disease germs from infected hands. It would also inculcate general ideas of the necessity of cleanliness and of the danger of transmitting disease through milk. The value of cleanliness in limiting the number of bacteria is demonstrated by the figures contained in the tables.

**General Conclusions.**—Because of its location and its hairy covering, the cow's udder is always more or less soiled with dirt and manure unless cleaned. On account of the position of the pail and the access of dust-laden air it is impossible to obtain milk by the usual methods without mingling with it a considerable number of bacteria. With suitable cleanliness, however, the number is far less than when filthy methods are used, there being no reason why fresh milk should contain in each cubic centimeter, on the average, more than 12,000 bacteria per cubic centimeter in warm weather and 5000 in cold weather. Such milk, if quickly cooled to 46° F., and kept at that temperature, will at the end of thirty-six hours contain on the average less than 50,000 bacteria per cubic centimeter, and if cooled to 40° F. will average less than its original number.

With only moderate cleanliness such as can be employed by anyone without adding appreciably to his expense, namely, clean pails, with small openings, straining cloths, cans or bottles, and hands, a clean place for milking, and a decent condition of the cow's udder and belly, milk when first drawn will not average in hot weather over 30,000, and in cold weather not over 25,000 bacteria per cubic centimeter. Such milk, if cooled and kept at 50° F., will not contain at the end of twenty-four hours over 100,000 bacteria per cubic centimeter. If kept at 40° F. the number of bacteria will not be over 100,000 per cubic centimeter after forty-eight hours.

If, however, the hands, cattle, and barns are filthy and the pails are not clean, the milk obtained under these conditions will, when taken from the pail, contain very large numbers of bacteria, even up to 1,000,000 or more per cubic centimeter.

Freshly drawn milk contains a slight and variable amount of bactericidal substances which are capable of inhibiting bacterial growth. At temperatures under 50° F. these substances act efficiently (unless the milk is filthy) for from twelve to twenty-four hours, but at higher temperatures their effect is very soon completely exhausted, and the bacteria in such milk will then rapidly increase. Thus the bacteria in fresh milk which originally numbered 5000 per cubic centimeter decreased to 2400 in the portion kept at 42° F. for twenty-four hours, but rose to 7000 in that kept at 50° F., to 280,000 in that kept at 65° F., and to 12,500,000 in the portion kept at 95° F.

As we have seen, the milk in New York City before general pasteurization was adopted was found on bacteriological examination to contain, as a rule, excessive numbers of bacteria. During the cold weather the raw milk in the shops averaged over 300,000 bacteria per cubic centimeter, during cool weather about 1,000,000, and during hot weather about 2,000,000.

The above statement holds for milk sold at the ordinary shops, and not that of the best of the special dairies, where, as previously stated, the milk contained only from 1000 to 30,000 bacteria, according to the season of the year. The committee on the bacteriological examination of milk of the A. P. H. A. at the meeting at Cincinnati in

October, 1916, presented the following statement of the interpretation to be placed on milk counts and the number of bacteria permissible.

1. Where the analysis can be made immediately after the milking the number of bacteria enables conclusions to be drawn as to the cleanliness and care in the dairy and the thoroughness in the cleaning and sterilization of the milk vessels, or sometimes the presence of cows with diseased udders. With properly cleaned and sterilized milk vessels and proper care in the farm and dairy the numbers of bacteria should not exceed 10,000, and may be easily brought down to 5000. Numbers beyond these in milk analyzed immediately after the milking may be regarded as an indication of unclean dairy methods, dirty and unsterile milking vessels, or to diseased udders. Apart from diseased udders the factors in dairying that most noticeably increase the bacteria count are unclean udders, milking with wet hands, unsterile milk vessels, unsterile strainers, and failure to cool the milk promptly.

2. If the milk is properly cooled with ice the numbers should not materially increase in five to seven hours. Communities within five to seven hours of their dairies should be able therefore to obtain milk with nearly as low a count as above indicated. Hence in such communities bacterial counts above these numbers should not be found in properly guarded milk. A count of 50,000 in such a community is an indication either of unsatisfactory dairy conditions or of failure to properly cool the milk during transportation. Night's milk if properly cooled can also easily be brought within these limits if analyzed the next morning. A count of over 50,000 for a community close to the dairies must be regarded as unsatisfactory, and the number should not be much more than 10,000 for high grade milk. In hot summer weather the difficulties of keeping low counts are greater, but even then they need not surpass 30,000 if the milk is properly cooled.

3. Where milk must be a longer time in transportation from the dairy there will be inevitably an increase in bacteria, depending on the length of time and the temperature. Experience has shown, however, that even in these conditions the excessively high numbers that have frequently been found in city milk are in reality due to dirty dairy conditions, to dirty and unsterile milk utensils, or culpable neglect in cooling. Moreover, such high bacterial counts at the shipping station are frequently traceable to a few dirty dairies whose milk with an abnormally high count contaminates the rest of the supply. Dirty shipping cases and warm temperatures in shipping are responsible for most of the high bacterial counts in city milk. Where the milk from healthy cows reaches the city within twenty-four hours, however, the number should not be over 100,000 in winter or 200,000 in summer, and numbers in excess of this may be regarded as due either to improper dairy conditions, dirty milk vessels, or insufficient cooling. In larger cities where much of the milk is forty-eight hours in reaching the city higher numbers may naturally be expected. But even under these conditions there is no good reason why the number of bacteria should reach 1,000,000; and it may mostly be brought down to below 200,000.



In such cities, therefore, milk with more than 1,000,000 bacteria must be regarded as improperly guarded either at the dairy or on its transit.

4. For a Grade A milk higher demands should be made than for the ordinary grade. The standard set by the Milk Commission for Grade A, viz., of 200,000 for milk to be subsequently pasteurized or for 100,000 to be used raw, is stated by that Commission to be an extreme limit for the most unfavorable conditions. Cities situated near the supplying dairies should demand a much higher standard, which should not allow over 10,000 in bacterial content in Grade A milk in communities favorably situated.

5. For communities situated where ice is not available it may be necessary to accept a milk with a higher bacterial content; but as rapidly as possible the standard should be made to approach the limits above given.

The question might be raised, Are even these enormous numbers of bacteria often found in milk during hot weather harmful?

Our knowledge is probably as yet insufficient to state just how many bacteria must accumulate to make them noticeably dangerous in milk. Some varieties are undoubtedly more harmful than others, and we have no way of restricting the kinds that will fall into milk, except by enforcing cleanliness. We have also to consider that milk is not entirely used for some twelve hours after being purchased, and that during all this time bacteria are rapidly multiplying, especially where, as among the poor, no provision for cooling it is made. Slight changes in the milk which to one child would be harmless, would in another produce disturbances which might lead to serious disease. A safe conclusion is that no more bacterial contamination should be allowed than it is practicable to avoid. Any intelligent farmer can use sufficient cleanliness and apply sufficient cold, with almost no increase in expense,  $\frac{1}{2}$  cent per quart, to supply milk twenty-four to thirty-six hours old which will not contain in each cubic centimeter over 50,000 to 100,000 bacteria, and no milk containing more bacteria should be sold.

The most deleterious changes which occur in milk during its transportation are now known not to be due to skimming off the cream or to the addition of water, but to the changes produced in the milk by multiplication of bacteria. During this multiplication, acids and distinctly poisonous bacterial products are added to the milk, to such an extent that much of the milk has become distinctly deleterious to infants and invalids. It is the duty of health authorities to prevent the sale of milk rendered unfit for use because of excessive numbers of bacteria and their products.

The culture tests to determine the number of bacteria present in any sample of milk require at least forty-eight hours; so that the sale of milk found impure cannot be prevented. It will, however, be the purpose of the authorities gradually to force the farmers and the middlemen to use cleanliness, cold, and dispatch in the handling of their milk, rather than to prevent the use of the small amount tested on any one day.

If the milk on the train or at the dealer's were found to contain excessive numbers of bacteria, the farmers would be cautioned and instructed to carry out the simple necessary rules furnished them on a printed form.

**Transmission of Contagious Diseases through Milk.**—**Pathogenic Bacteria in Milk.**—Tuberculosis, typhoid fever, septic sore throat, scarlet fever, and diphtheria are the chief diseases transmitted by means of milk in this locality. In other countries cholera, Malta fever, and possibly other diseases may be due at times to milk infection. The obscure disease *trembles* is also believed to be due to milk.

The tubercle bacilli are in the majority of cases derived from the cow, but may come from human sources, the typhoid bacilli are entirely from man, the contagion of true scarlet fever conveyed in milk is probably always from man, but the contagion of a disease closely allied to it is certainly given off by cows suffering from certain septic diseases as yet not fully identified. Diphtheria bacilli are probably always of human origin, as animals, except cats, practically never suffer from the disease and these only under exceptional conditions. The streptococci exciting septic throats are probably usually from human cases of septic inflammation but may at times come from septic cows. As milk is usually kept below 60° F. the typhoid bacilli and the streptococci are the only pathogenic germs that we believe increase in any appreciable extent.

The following epidemics and cases have been recorded in the bulletin of the Marine Hospital Service, as produced by cow's milk:

	Epidemics.	Cases.
Typhoid fever . . . . .	179	6900
Scarlet fever . . . . .	51	2400
Sore throat . . . . .	7	1100
Diphtheria . . . . .	23	960
Tuberculosis . . . . .	...	....

No case of measles, smallpox, chicken-pox, whooping-cough, mumps or poliomyelitis has been clearly traced to milk.

**The Relation of the Typhoid Carrier to Milk Infection.**—Many epidemics of typhoid fever have until recently puzzled investigators because, though evidently milk-borne, yet no case of typhoid fever could be found. The discovery that about 2 per cent. of those who have recovered from typhoid fever remain infected and continue during the rest of their lives to pass typhoid bacilli has cleared up the mystery. Epidemics due to these carriers have already been traced both in New York City and elsewhere. Many observers have already discussed the relation of typhoid cases to milk infection. Hands, water, flies, etc., may all aid in the transfer of the bacilli from the dejecta to the milk. Recently we traced over 400 cases to infection of a milk supply by a typhoid carrier who had the disease forty-seven years ago.

**The Conveyance of Scarlet Fever by Means of Milk.**—As we do not know the organism which excites scarlet fever, we are not as clear as to the means by which it is spread as we are in the case of tuberculosis, typhoid fever, and diphtheria. We know, however, that the throat

secretions are dangerous. Where the infection has been traced it has usually been found that the milker has suffered from an unrecognized case or is convalescent. It seems as if the contagion must either increase in milk or be capable of infecting when greatly diluted, for cases have developed from milk after great dilution. A small number of epidemics have appeared to come from the milk of diseased cows. Many are skeptical about this, but after personal experience we think it probable. The history of one case was as follows: The milk from a septic cow was delivered to two schools. About thirty of the boys who drank the milk developed the disease while none of the day scholars who went home to lunch did. Some of the cases developed at first only sore throats, others only the rash. On the second day the cases resembled very closely scarlet fever. There was no scarlet fever in the town. The milk contained immense numbers of long-chained streptococci.

Diphtheria and septic sore throats are occasionally produced by milk. The diphtheria bacilli usually originate from a mild case, the nature of which is not detected. Septic sore throats produced by milk are usually traced to contamination from a human source, but like cases developing the scarlet rash the infection may come from cows suffering from some acute septic udder disease.

**The Grading of Milk.**—The appreciation of the importance of the bacteria in milk has led to the pasteurization of all milk entering New York City, except that produced and transported under the very best conditions. Together with the farm conditions the bacterial content of milk is used to grade it. All milk in New York City is divided into grades A, B, and C.

Grade A is raw and pasteurized. The raw is from cows which have successfully passed the tuberculin test.

Grade B is all pasteurized.

Grade C is all pasteurized.

The grade A may contain 60,000 bacteria per cubic centimeter.

The grade B may contain 1,500,000 when raw and 50,000 after pasteurization.

The grade C may contain any number within reason before and 100,000 after pasteurization. It is supposed to be used for cooking purposes only. The State of New York has adopted a similar grading except that it allows raw and pasteurized in all three grades. Many cities and States are adopting such grades. A powerful influence in this direction was the report of the national committee of experts on milk standards. This was a commission appointed by the New York Milk Committee.

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## CHAPTER L.

### THE BACTERIOLOGICAL EXAMINATION OF SHELLFISH.

Of the shellfish commonly used as food, oysters are the most extensively eaten. According to the United States Deputy Commissioner of Fisheries (1913): "Economically, oysters are the most important of all cultivated water products." He estimates the entire oyster crop of the world as over 42,000,000 bushels, representing a money value of \$25,000,000. The share of the United States in this industry is about 88 per cent. of the quantity and about 70 per cent. of the value. Three-fourths of this is controlled by the following States: New York, Virginia, Connecticut, Massachusetts, Maryland, New Jersey, Rhode Island and Louisiana.

In their normal habitat, in sea waters free from pollution, shellfish are free from dangerous bacteria, but since cities and towns situated on or near the sea coast find it convenient and advantageous to use running water for the disposal of their sewage, oyster-breeding grounds may be subject to pollution and the oysters infected with organisms of the intestinal type. Only when the pollution is sufficiently remote is serious contamination of the beds avoided and this can only be determined by careful sanitary and bacteriological examinations. As a matter of fact, oysters should not be marketed or harvested from waters which are exposed to dangerous sewage pollution.

Serious epidemics of typhoid due to the eating of infected shellfish have been reported at various times. One of the first outbreaks which called attention to this danger was reported by Professor Conn, of Wesleyan University. Investigations showed that the oysters had been fattened at the mouth of a stream, that a house nearby contained two cases of typhoid fever and that drainage from the house entered the stream.

The period of infection of oysters is transitory, however. If they are removed from polluted waters, they will cleanse themselves and be safe for food in from six to eight days. This is done at Concarneau, France. Sea water is raised mechanically, is passed through coarse filters, then through sand and finally into reservoirs containing the oysters. Here the water is slowly changed and the purification of the oysters results.

Artificial purification of oysters from polluted beds has been reported by Wells, and the method is being tried out on an extensive scale in the harbor at New Haven, Conn. The oysters are placed in water-tight stationary floats containing sea water. Hypochlorite of lime is added in about the proportion of one part of available chlorine to one hundred thousand parts of water. The water is treated a second time with the

hypochlorite, about six hours later. This is to insure the disinfection of organisms which may have escaped the first treatment, by reason of the closing of the oysters when the disinfectant was added until such time as the water is no longer objectionable to them. In twenty-four hours, the total number of organisms in the oysters is greatly reduced and there are practically no bacteria of the *B. coli* group.

It has been noticed by several observers that there is a seasonal variation in the bacterial content of oysters. Gorham (1912) in a series of examinations, found that results obtained from oysters examined in the summer did not agree with those obtained from oysters from the same beds examined in the winter. He concluded, therefore, that oysters hibernate.

Experiments carried on in the Research Laboratory of the New York City Health Department (1912) showed that oysters placed in typhoid-infected sea water did not become infected with typhoid bacilli while the temperature was maintained at 3° C. The surrounding water contained 13,000 typhoid bacilli per cubic centimeter. Further experiments along the same lines were made by Pease. He placed oysters and sea water in separate containers in the ice-box and held them at a temperature of 36° F. overnight. Then fuchsin was added to the water and the oysters were placed in the solution. The whole was left in the ice-box all day. Another lot of oysters was placed in fuchsin sea water and kept at a temperature of 65° F. Both sets of oysters were washed in salt water to remove the fuchsin from the shells. When opened the oysters which had been kept at 34° F. showed no trace of fuchsin, while the gills of the oysters kept at 65° F. were turned a dark fuchsin color.

From these experiments he concluded that oysters kept at a temperature of 34° to 36° F. will remain closed, and that since particles of soluble dyes in aqueous solution are much smaller than the bodies of bacteria, bacteria in the waters surrounding the oysters are totally excluded. Furthermore, he says, "Those organisms which have previously gained access to the oyster are destroyed or gradually eliminated so that the total number of bacteria in the oysters is greatly reduced and the oysters become practically free from colon bacilli. Oysters gathered during the hibernating season are more easily handled than during the early part of the oyster season."

**Standard Methods for the Examination of Shellfish Adopted by the American Public Health Association.—Oysters in the Shell.—Selection of Sample.**—Twelve oysters of the average sizes of the lot under examination, with deep bowls, short lips and shells tightly closed, shall be picked out by hand and prepared for transportation to the laboratory. As complete a record of such data as is possible to obtain shall be made covering the following points: The exact location of the bed from which the sample has been selected. The depth of the water over the bed at time of collection. The state of the tide. The direction and velocity of the wind. Other weather conditions. The day and hour of the removal of the stock from the water. The conditions under which the stock has been kept since removal from the water and prior to the taking of the sample. The day and the hour of the taking of the sample.

**Transportation of the Sample.**—The oysters so selected shall be packed in suitable metal or pasteboard containers of such size and shape that a number

of them can be enclosed in a shipping case capable of satisfactory refrigeration by means of ice. The important points in this connection are:

A. The prevention of the mixing of the oyster liquor of different samples, and of the mixing of the ice-water with the oysters.

B. The icing of the samples if they are not to arrive at the point of laboratory examinations inside of thirty-six hours or if the outside temperature is above 50° F.

It is not necessary to enclose the oysters in an absolutely tight container providing the above conditions are maintained.

*Condition of Samples.*—Record shall be made of the general condition of the oysters when received, especially whether the shells are open or closed; of the presence of abnormal odors and of the temperature of the stock.

*Technical Procedure.*—The bacteriological examination shall be started as soon as possible after the receipt of the sample.

The oysters shall be thoroughly cleaned with a stiff brush and clear running water and then dried. The edges of the shell shall be passed through the flame or burned with alcohol.

The opening of the shell shall be accomplished by either of the following methods:

A. By the use of a sterile oyster knife in the usual manner.

B. By drilling through a flamed portion of the shell near the hinge with a sterile drill. The drill shall be sterilized and the site of the operation the shell be flamed at least once during the drilling process.

*Bacterial Counts.*—Bacterial counts shall be made of the composite sample of each lot obtained by mixing the shell liquor of five oysters. Agar shall be used for the culture medium and in general the procedure shall be in accordance with the method recommended for examination of water by the Committee of Standard Methods of Water Analysis of the American Public Health Association.

The water used for dilution purposes shall contain 1 per cent. sodium chloride, in order to approximate the natural salinity of oyster liquor.

The agar plates shall be incubated at 20° C. for three days and the colonies then counted.

A satisfactory method of opening an oyster is to strike it a sharp blow with a hammer directly on the large muscle which holds the shells together. This injures the oyster just enough to cause the shells to spring apart. The edges of the shell are passed through the flame and the liquor is poured into a sterile test-tube.

**Determination of Bacteria of the *Bacillus Coli* Group.**—The quantitative determination of the presence of *B. coli* shall be in accordance with the following procedure:

Measured quantities (1.0, 0.1, 0.01 c.c., etc., or their equivalents in dilutions) of the shell water of each of five oysters selected from the dozen, shall be placed in fermentation tubes containing lactose peptone bile, prepared according to the method recommended by the Committee on Standard Methods of Water Analysis. These shall be incubated for three days at 37° C., and the presence or absence of gas noted daily. For all ordinary purposes of routine work, a development of 10 to 85 per cent. of gas during this time period shall constitute a positive test indicating a presumption of the presence of at least one bacterium of the *Bacillus coli* group in the quantity of shell water tested. But no final *B. coli* rating based on these results shall be used for official<sup>1</sup> approval or condemnation unless positive confirmatory tests for the presence of organisms of the *B. coli* group shall have been obtained from the tube of highest or next highest dilution from each oyster; showing the presence of gas. These confirma-

<sup>1</sup> There seems to be no special object gained in confirming the presumptive tests in oysters showing low scores. A low score is approved anyway and confirmation of *B. coli* will not raise the score.

tory tests shall be begun immediately upon noting the formation of gas, and carried out in accordance with the procedure recommended by the Committee on Standard Methods of Water Analysis.

**Statement of Results.**—The results of the bacterial counts shall be expressed as number of bacteria per cubic centimeter. The results of the lists for *B. coli* shall be expressed either in the form of the following arbitrary numerical system to be known as "The American Public Health Association Method of Rating Oysters for *B. coli*"; or in Estimated Number of Bacteria of the *B. coli* Group per Cubic Centimeter of the Sample."

The presence of *B. coli* in each oyster of the five examined is to be given the following values, which represent the reciprocals of the greatest dilutions in which the test for *B. coli* is positive:

If present in 1 c.c. but not in 0.1 c.c., the value of 1. If present in 0.1 c.c. but not in 0.01 c.c., the value of 10. If present in 0.01 c.c. but not in 0.001 c.c. the value of 100, etc.

The addition of these values for the five oysters would give the total numerical value for the sample, and this figure would be the score for *B. coli*.

The results should be expressed in the following tabular form:

RESULTS OF TESTS FOR *B. COLI* IN DILUTIONS INDICATED.

Oysters.	1 c.c.	0.1 c.c.	0.01 c.c.	Numerical value.
1	+	+	0	10
2	+	+	0	10
3	+	0	0	1
4	+	0	0	1
5	+	0	0	1

Total or score for *B. coli* = 23

+ = Presence of *B. coli* group in fermentation tube test with lactose bile where subsequent isolation tests have confirmed the results of the presumptive test or other satisfactory test.

0 = Failure to demonstrate presence of *B. coli* group.

**Estimated Number of *B. Coli* per Cubic Centimeter.**<sup>1</sup>—It will be seen that if the *B. coli* score is divided by 5, the standard number of oysters tested, the results will approximate the number of *B. coli* per cubic centimeter of shell water. Partly because it does not do this exactly but also for simplicity and the avoidance of fractions, the method of stating results as an arbitrary "score" is preferred by the committee. Practical experience with the method has also appeared to justify this conclusion.

Sometimes results similar to the following are obtained, that is, one or more oysters may show positive results in small quantities of shell water while an equal number may show negative results in larger quantities. In this case the next lower numerical value should be given to the positive results in the high dilutions and such positive results should be considered as being transferred to a lower dilution giving negative results in another oyster. This is done in order to avoid the unnatural result that could follow from what is probably an unequal distribution of the bacteria in the shell water. This recession of numerical values, however, should not be carried beyond the point where the number of such recessions is greater than the number of instances where other oysters in the series failed to give positive *B. coli* results.

As an example of the method of obtaining the score for *B. coli*, the following illustration is given.

<sup>1</sup> Where the term *B. coli* is used it refers in all cases to bacteria of the *B. coli* group and not to the specific prototype.

## RESULTS OF B. COLI TESTS IN DILUTIONS INDICATED.

Oysters.	1 c.c.	0.1 c.c.	0.01 c.c.	Numerical value.
1	+	+	0	10
2	+	+	0	10
3	+	+	0	10
4	+	0	0	10 (Not 1)
5	+	+	+	10 (Not 100)

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Score, 50

**Examination of Oysters Removed from the Shell, or Shucked Stock.**

—The procedure specified for oysters in the shell shall be followed, but attention is called to the fact that higher dilutions than 100 c.c. are usually required. Triplicate fermentation tubes shall be inoculated from end dilutions of the sample.

*Statement of Results.*—The results of the bacteriological examination of the opened oysters or shucked stock shall be expressed in the same way as that specified for oysters in the shell, except that in the calculations of B. coli, rating the values for the results of the fermentation tests after confirmation shall be recorded for each of the inoculations of each dilution. In order that the rating from these triplicate tests may be compared with that obtained from testing five oysters in the shell, the sum of the values for the triplicate tests shall be multiplied by  $\frac{5}{3}$ . If, instead, the sum is divided by 3, the result will give approximately the number of B. coli per cubic centimeter.

**Clams and Other Shellfish.**—The methods for examining clams and shellfish other than oysters shall be those given above. Certain modifications are necessary in the method of handling the sample and the opening of the shells, etc.

Clams are more likely to lose water during transportation than oysters. It is therefore necessary to take greater precautions to separate different samples of clams from each other than in the case of oysters.

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## CHAPTER LI.

### THE SOIL BACTERIA AND THEIR FUNCTIONS. SEWAGE BACTERIA. BACTERIA IN INDUSTRIES.

#### SOIL BACTERIA.

THE bacteria in the soil belong to many varieties. Some varieties are only accidentally present, being due to the contamination of the earth with the bacteria contained in animal feces and other waste products. The majority, however, pass their life and reproduce themselves chiefly or wholly in the soil. Many of these varieties have most important functions to perform in continuing the earth's food supply. Without them plant food, and therefore animal food, would cease to exist. Some make available for plants the carbon, nitrogen, hydrogen, and other compounds locked up in the dead bodies of animals and plants. Others construct food for plants from the gases of the air and the inorganic elements of the earth which in their simpler forms were not available.

The bacteria, together with the other somewhat less important microscopic plants and animals, thus form a vital link in the earth's life cycle of plants and animals. The bacteria in the soil require for their activities food, moisture, and a proper temperature. They may be present to the extent of many millions in a single gram of rich loam, while in an equal quantity of sand they may be almost absent.

The various species associated together in the soil flora influence each other. Thus anaërobic bacteria are enabled to grow because of associated aërobes using up the free oxygen, while other species make assimilable substances not usable by others.

**The Splitting up of Carbon Compounds.**—The plants form starch, and from it cellulose, wood, fats, and sugar. These substances once formed cannot be utilized by other generations of plants. Some of these are transformed in the bodies of animals, but the largest percentage await the activities of the microorganisms. The sugars and starches usually undergo an alcoholic fermentation excited by the yeasts and molds with the production of alcohol and carbon dioxide, or an acid fermentation excited by bacteria with the production of acids and frequently of carbon dioxide.

Cellulose which is so resistant to decay is attacked by certain varieties of bacteria which are abundant in the soil. They act both in the presence and absence of free oxygen. Molds also act on cellulose. Carbon dioxide, marsh gas, and other products are produced. Wood is apparently first attacked by the fungi and only later by other microorganisms. These bacteria are carried into the intestines and act upon cellulose and other substances.

**The Decomposition of Nitrogenous Compounds.**—Plants obtain their nitrogen chiefly in the form of nitrates. The small amount of usable nitrogen in the soil must be constantly replenished. This must either come from the nitrogen forming a part of protein materials or from the free nitrogen in the air.

The animals utilize the plant proteins and reduce them to much simpler compounds, such as urea, but even these are not suitable for plant use. We now know that microorganisms are employed to break compounds into simpler compounds and also to utilize the nitrogen of the air.

**Decomposition.**—This process is to some extent carried out through the agency of molds and other fungi but it is chiefly due to the activities of bacteria. When this process is carried on in the absence of oxygen it is incomplete, giving rise to substances with unpleasant odors, such as  $H_2S$ ,  $NH_3$  and  $CH_4$ . This is called putrefaction. When oxygen is freely accessible more complete decomposition occurs with such end-products as  $CO_2$ ,  $N$  and  $H_2O$ . These two processes, putrefaction and complete decay, cannot be sharply separated, as the second usually follows the first. The varieties of organisms causing these changes are many. Some groups will be found chiefly in decaying vegetable substances, others in animal tissues. They include all morphological forms of bacteria as well as yeasts and higher fungi. These forms exist everywhere in nature, although in various degrees, so that every bit of dead organic matter is sure to be decomposed if only moisture and warmth are present. The *B. subtilis* and *B. proteus vulgaris* groups are well-known laboratory bacteria that are commonly found among decomposing materials. *B. proteus* is described under Pathogenic Bacteria. *B. subtilis*, or hay bacillus (type form), has the following characteristics (Fig. 208):

**Source and Habitat.**—Hay, straw, soil, dust, milk, etc.

**Morphology.**—Short, thick rods with round ends, sometimes form threads; sometimes also chains of long rods, short rods, and coccus forms.  $0.8\mu$  to  $1.2\mu$  broad,  $1.3\mu$  to  $3\mu$  long. Often united in strings and threads.

**Staining Reaction.**—Stains by Gram's method.

**Capsule, Flagella, Motility.**—Bacilli possess a thin capsule and many flagella which are long and numerous; short forms actively motile; threads immotile.

**Spore Formation.**—Oval spores formed in presence of air germinating at right angles to long diameter. Spores are set free in about twenty-four hours, size  $1.2\mu$  by  $0.6\mu$ ; widely distributed in nature, dust, air, excreta, etc. (Plate III, Fig. 23).

**Biology: Cultural Characters (Including Biochemical Features).**—**Bouillon.**—Uniformly cloudy growth with marked pellicle, wrinkled and thick; copious spore formation.

**Gelatin Plates and Tubes.**—Saucer-like depressions; colonies have granular centres and folded margins. Surface growth in stab cultures is whitish-gray; colonies sink on liquefaction of medium; liquefaction progresses in a cylindrical form, and a thick white scum is formed.

**Agar Plates and Tubes.**—Small, irregular, grayish-white colonies; moist glistening growth along needle track in stab cultures.

The bacteria in taking certain atoms from the molecules utilized in their growth leave the other atoms to enter into new relations and

form new compounds. The actual products will depend on the decaying substance, the variety of bacteria and the conditions present.

**Nitrification.**—This is a process of oxidation by which through bacterial activities ammonia compounds are changed to nitrates and thus rendered utilizable by plants. This change is accomplished in two stages: first, the ammonia is oxidized to nitrite and, second, to nitrate. The nitrates are taken up by the plant roots from the soil. The bacterial nature of these changes were discovered in 1877 by two French investigators, Schlosing and Muntz. They noted that fermenting sewage after a time lost its ammonia and gained in nitrates, but that if the sewage was treated with antiseptics, so that fermentation ceased, no such change occurred. Warrington first and Winogradsky later more thoroughly investigated the bacterial cause of these changes. The latter by means of silica jelly, which contained no organic matter, was able to isolate two varieties of cocci, one in Europe and the other in America, which were able to change ammonia to nitrites. He called

the one *nitrosomonas* and the other *nitrosococcus*. They are capable of acting on almost any ammonia salt. One variety of organisms capable of changing nitrites to nitrates was isolated, and this bacillus he called *nitrobacter*. These are small, slightly elongated bacilli. These bacteria are remarkable in that in pure cultures very small amounts of organic matter in the media act as antiseptics. They appear to be able to depend on mineral substances for their food. These bacteria are extremely important, for the plants take up most of their nitrogen in the form of nitrates. These changes are mostly produced in the surface soil. If the reaction of the

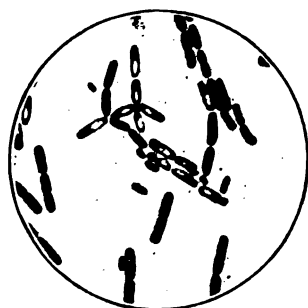


FIG. 207.—*Bacillus subtilis* with spores. Agar culture. Stained with gentian violet.  $\times 1000$  diameters. (Fränke.)

soil becomes acid growth ceases. Soil bacteriologists are studying the nitrifying power of different types of soil under identical conditions. The process being one of oxidation, the access of air is necessary.

**Denitrification.**—This is a reducing process. The nitrate is made to yield up a part or all of its oxygen and thus becomes changed to nitrites and to ammonia and even to free nitrogen. The partial change does not rob the soil of its available nitrogen as does the total change, for the nitrites and ammonia may be changed by the nitrifying bacteria to nitrates. These bacteria exist normally in most soils and are especially abundant in manure. There are three different types of nitrogen reduction: (1) The reduction of nitrates to nitrites and ammonia. (2) The reduction of nitrates and nitrites to gaseous oxides of nitrogen. (3) The reduction of nitrites with the development of free nitrogen gas.

**Nitrogen-fixing Bacteria.**—Helbrigel, in 1886, demonstrated that certain plants were able to use the nitrogen of the air and this apparently through the aid of bacteria growing in their roots. These root bacteria

are named *B. radiculicola*. They produce enlargement (tubercles) on the roots.

According to Ball, there is no reasonable doubt but that *B. radiculicola* can and usually does remain active for very long periods in soil devoid of leguminous vegetation. Furthermore, the bacterium diffuses at a very considerable rate through soils that are in proper condition; therefore if a soil should be found lacking the organism, it is illogical to attempt to introduce it artificially without having first made the soil fit for the development of the bacteria.

It has not been shown by anyone that increased powers of resistance to unfavorable conditions of certain varieties are at all correlated with their enhanced "greed for nitrogen." Moreover, it is far from being proven that any one race or "physiological species" is really more virile than another. Greig-Smith has shown that as many as three races are sometimes present in one and the same tubercle. Possibly, therefore, fixation of nitrogen may occur most rapidly only when two or more of these races are growing together.

Buchanan has recently made a minute morphological study of *B. radiculicola*. Some of his conclusions are as follows:

1. Considerable variation in the morphology of *B. radiculicola* may be induced in artificial media by the use of appropriate nutrients. Of the salts of the organic acids, sodium succinate brings about the most luxuriant development and the production of the greatest variety of bacteroids.

2. *B. radiculicola* in the roots of the legumes shows the same type of bacteroids as may be found in suitable culture media. On the other hand, there is little or no correspondence between the type of bacteroid produced in culture media by a certain organism and that produced in the nodule by the same form.

3. It is probable that the term *B. radiculicola* includes an entire group of closely related varieties or species, which differ from each other to some degree in morphological characters.

4. The nodule organism resembles morphologically both the yeasts and the bacteria. The difference between this form and those ordinarily included under the terms *Bacillus* and *Pseudomonas* justify the use of a separate generic name, *Rhizobium*.

In 1893 Winogradsky furnished proof that there are in the soil, bacteria which are outside of the plant roots performing the same function as those within the roots. These bacilli he called *Clostridium pasteurianum*. They are anaërobic and produce spores. Their power to fix nitrogen is increased in presence of sugar and lessened in presence of nitrogenous substances.

Beijerinck, in 1901, described two aërobic species of nitrogen-fixing bacteria. Later Bailey described three additional species. These were called *Azotobacter*. These studies have already led to the inoculation of soils and to the investigation of the kind of soils and crops best fitted for the growth of these bacteria. Many impoverished soils have already been greatly improved. There are probably many other varie-

ties of bacteria capable of fixing nitrogen, because one can hardly examine the roots of any leguminous plants without finding different kinds of tubercles. The use of seed inoculated with the special variety of bacteria suitable for the plant and the soil is already largely practised.

**Bacteria and Soil Minerals.**—Some of the bacterial products act upon the inorganic constituents of the soil. The carbon dioxide and the organic acids act upon compounds of lime and magnesia, practically insoluble in water, to form more soluble substances. The same is true of the rock phosphates, the silicate of potassium, sulphates, etc.

Scientific farming is beginning to make use of the knowledge already acquired, and there is reason to hope that great practical advantages will flow from the investigation of the relation of bacteria to soil exhaustion and replenishment.

The effect of excessive bacterial development appears at times to be harmful to the soil. Each crop seems to favor the growth of certain varieties, and the exhaustion of the soil which follows the constant raising of the same crop is now suspected to be due in part at least to the continuance of a few restricted species of bacteria in the soil, which failing to produce all the necessary substances for the nutrition of the special crop, vegetation suffers, or again the bacteria finally entirely dissipate substances already in the soil necessary to growth.

The application of manure not only adds food for plant life, but also countless numbers of bacteria which make the food more available. The greatest number of bacteria are contained a little below the surface of the soil, where they are protected from drying and sunlight and are in contact with oxygen and with the roots and other food of the superficial soil.

### BACTERIA IN SEWAGE.

The materials which flow from our sewers are a menace to public health, mainly because they so frequently contain pathogenic bacteria. The other products of men and animals are offensive, but rarely concentrated enough in drinking water to be appreciably deleterious. Sewage can be made harmless by being sterilized, but can be freed from offense only by the destruction of organic matter. This, except when chemical precipitants are used, is almost wholly obtained through bacterial processes. The purifying value of soil has long been recognized. This is largely due to the action of the soil bacteria.

In 1895 the Englishman, Cameron, introduced the "septic tank," which was a covered cemented pit. The sewage admitted at the bottom flowed out at the top, after about twenty-four hours' subjection to anaërobic conditions. The anaërobic bacteria during this time ferment the organic matter energetically, liquefy it, and develop abundant gas. The knowledge that soil and sand filters act not only mechanically but also and perhaps chiefly bacteriologically, having been acquired, intermittent soil filtration was established as one of the best means of bacteriologically purifying sewage. The sewage is conducted to the beds, allowed to pass through, and then after a few hours again poured

on. The purification is based chiefly on the action of the aërobic bacteria in the upper layers of the soil or sand. The best practical results are obtained by combining the two processes: first the anaërobic treatment is used to break down the solid materials, and then the intermittent sand filtration, to oxidize the compounds and render these products harmless. With low temperatures the chemical changes are very much lessened and the filter beds act more like pure mechanical filters.

The anaërobic bacteria change the protein substances into simple chemical compounds, among which is ammonia. The carbohydrates are changed into gaseous compounds, acids, etc. The gases are mainly nitrogen, carbon dioxide, and marsh gas. The bacterial changes produced in sewage poured on contact beds made of coarse coke, clinkers, or other material act much as in the sand filters after the filtration.

**Varieties of Bacteria in Filter Beds and Septic Tanks.**—The septic tanks all contain spore-bearing bacilli which destroy cellulose, others that attack nitrogenous compounds. The cocci are in a minority. The filter beds have a number of small, non-spore-bearing bacilli; some of these change ammonia into nitrites and nitrates. There are also denitrifying bacteria. As before mentioned, the bacterial efficiency of the bed is increased with suitable temperature and much lessened with low temperature.

**Sewage Farming.**—The action of bacteria is utilized in the breaking up of sewage which has been distributed over fields. The amount of sewage which can be poured on a certain area is limited. One acre of land can usually take care of the sewage from one hundred persons. If too much is poured on, it runs off impurified or clogs the soils and prevents the access of oxygen to aërobic bacteria. In warm weather evaporation and bacterial activities are much greater than in cold weather. So far as experience shows, those who eat vegetables from these small farms contract no disease from them.

### THE PRESERVATION OF FOODS AGAINST DECOMPOSITION BY MICROÖRGANISMS.

The preservation of foods against decomposition by bacteria, yeasts, molds, and higher fungi is obtained by using processes which will prevent the growth of microörganisms. Drying, exposure to wood smoke with consequent absorption of creosote, the addition of salt and sugar, of acids such as vinegar, spices, germicides such as boracic acid, formaldehyde, all are familiar methods of making foods unsuitable for bacterial growth.

Instead of using food preserved by drying or chemicals, products may be kept at temperatures too low for bacterial growth. Cold storage of meats, eggs, vegetables, etc., is now common.

The sterilization of food substances by heat with protection from infection afterward is made use of extensively in the canning of fruits and vegetables. Care must be taken that absolutely all bacteria are killed, for otherwise decomposition will finally occur.

**Bacterial Fermentation in Relation to Miscellaneous Products.**—Pasteur, in 1857, explained the process of fermentation as due to the action of microorganisms. He demonstrated that the change of sugar into lactic acid only occurred when living bacilli were present. If the fluid was sterilized the fermentation ceased. He stated that "organic liquids do not alter until a living germ is introduced into them." When the action is direct we speak of an organized ferment; when it is indirect, that is, due to the cell product, we call it an unorganized soluble ferment or enzyme. Similar enzymes are produced by the cells of the animal tissues, such as ptyalin, pepsin, and trypsin. Pasteur's work led to the conclusion that the different fermentations were due to different varieties of organisms. The major part of fermentation is due to yeast. Some important fermentations are due to bacteria and a few to the molds.

**Wines and Beers.—Alcoholic Fermentation.**—If there is a development of the yeast cells in a solution of grape-sugar we have a fermentation of the sugar with a final development of alcohol and carbon dioxide. It is thus that beers and wines are developed. When the carbohydrate is in the form of starch this is first converted into sugar and then later into the final products. If the sugar is in the form of saccharose, it is first changed by the yeast ferments to glucose. In all these three forms of fermentation the sugar is changed into alcohol and carbonic acid. When the alcohol reaches about 13 per cent. it stops further fermentation. These yeasts called *saccharomyces* comprise a number of distinct varieties, some of which are cultivated while others, called "wild yeasts," propagate themselves. The distillery, brewery, and wine industries each makes use of special yeasts and special conditions. The rising of bread is one of the most common uses of fermentation by yeast. The yeast acts upon the sugar made by the diastase from the starch. The resulting  $\text{CO}_2$  and alcohol create myriads of little bubbles in the dough.

**Diseases of Beer and Wines.**—Hansen, Pasteur, and others demonstrated that the spoiling of beers and wines was due to the development of varieties of bacteria and yeasts which produce different kinds of fermentation from that desired. These produce alterations in flavor, bitterness, acidity.

**Vinegar Making.**—Vinegar is made from some weak alcoholic solution by the union of alcohol with oxygen. This oxidation can be brought about by a purely chemical process. When vinegar is formed in the usual way bacteria are essential. The scum on the surface of the fermenting alcohol is a mass of microorganisms. The mother of vinegar was named *mycoderma* by Pearson. Kützing showed that this was composed of living cells. Hansen proved these to be bacteria. We now know there are many varieties of bacilli capable of producing this fermentation. Each variety has its own optimum temperature and differs in the amount of acid it produces. Most of these have the peculiarity of growing at high temperatures into long threads without any traces of division. At low temperatures they produce long threads

with swollen centres. The usual vinegar is made by using the variety of bacilli prevalent in the surroundings, but the custom is growing of adding to the pasteurized alcoholic solution the special variety desired in pure culture.

**Sauerkraut.**—This is cabbage leaves shredded, slightly fermented, and prevented from decay by the lactic acid bacteria. At first both yeasts and bacteria increase together, but with the increase in acidity all growth ceases. Putrefaction is prevented by the same cause. The lactic acid bacteria are the same as those found in sour milk.

**Ensilage.**—The fermentation is believed to be due partly to enzymes in the corn tissues and partly to bacterial action. The first changes are due chiefly to the enzymes.

**The Curing of Tobacco.**—The curing of tobacco is apparently due partly to bacterial processes and partly to the action of leaf enzymes.

**The Bacterial Diseases of Plants.**—These are probably as serious and varied for plants as for animals. The pear blight, the wilt disease of melons, the brown rot of tomatoes, the black rot of cabbages are examples. These plant diseases can be communicated by means of the pathogenic pure cultures of bacteria experimentally just as readily as animal diseases by their specific bacteria.

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## CHAPTER LII.

### THE DESTRUCTION OF BACTERIA BY CHEMICALS. PRACTICAL USE OF DISINFECTANTS.

MANY substances, when brought in contact with bacteria, combine with their cell substance and destroy the life of the bacteria. While in the vegetative stage bacteria are much more easily killed than when in the spore form, and their life processes are inhibited by substances less deleterious than those required to destroy them.

Bacteria, both in the vegetative and in the spore form, differ among themselves considerably in their resistance to the poisonous effects of chemicals. The reason for this is not wholly clear, but it is connected with the structure and chemical nature of their cell substance.

Chemicals in sufficient amount to destroy life are more poisonous at temperatures suitable for the best growth of bacteria than at lower temperatures, and act more quickly upon bacteria when they are suspended in fluids singly than when in clumps, and in pure water rather than in solutions containing organic matter. The increased energy of disinfectants at higher temperatures indicates in itself that a true chemical reaction takes place. In estimating the extent of the destructive or inhibitive action of chemicals the following degrees are usually distinguished:

1. The growth is not permanently interfered with, but the pathogenic and zymogenic functions of the organism are diminished—*attenuation*. This loss of function is usually quickly recovered.

2. The organisms are not able to multiply, but they are not destroyed—*antiseptic* action. When transferred to a suitable culture fluid free of the disinfectant these bacteria are capable of reproduction.

3. The vegetative development of the organisms is destroyed, but not the spores—incomplete or complete sterilization or disinfection, according as to whether spores are present in the organisms exposed and as to whether these spores are capable of causing infection.

4. Vegetative and spore forms are destroyed. This is complete *sterilization* or *disinfection*.<sup>1</sup>

The methods employed for the determination of the germicidal action of chemical agents on bacteria are, briefly as follows:

If it is desired to determine the minimum concentration of the chemical substance required to produce complete inhibition of growth we proceed thus: A 10 per cent. solution of the disinfectant is prepared and 1 c.c.,

<sup>1</sup> Disinfection strictly defined is the destruction of all organisms and their products which are capable of producing disease. Sterilization is the destruction of all saprophytic as well as parasitic bacteria. It is not necessary in most cases to require disinfectants to be capable of sterilizing infected materials containing spores, for there are but few varieties of pathogenic bacteria which produce spores.

0.5 c.c., 0.3 c.c., etc., of this is added to 10 c.c. of liquefied gelatin, agar, or bouillon, or, more accurately 10 c.c. minus the amount of solution added, in so many tubes. The tubes then contain 1 per cent., 0.5 per cent., 0.3 per cent., and 0.1 per cent. of the disinfectant. The fluid medium in the tubes is then inoculated with a platinum loopful of the test bacterium. The melted agar and gelatin may be simply shaken and allowed to remain in the tubes, and watched for any growth which takes place, or the contents of the tubes may be poured into Petri dishes, where the development or lack of development of colonies and the number can be observed. If no growth occurs in any of the dilutions, lower dilutions are tested. Bacteria that have been previously injured in any way will be inhibited by much weaker solutions of chemicals than will vigorous cells. The same test can be made with material containing only spores.

If it is desired to determine the degree of concentration required for the destruction of vegetative development, the organism to be used is cultivated in bouillon, and into each of a series of tubes is placed a definite amount of diluted culture from which all clumps of bacteria have been filtered; to these a definite amount of watery solution of different percentages of the disinfectant is added. At intervals of one, five, ten, fifteen, and thirty minutes, one hour, and so on a small platinum loopful of the mixture is taken from each tube and inoculated into 10 c.c. of fluid agar or gelatin, from which plate cultures are made. Whenever it is probable that the antiseptic power of the disinfectant approaches somewhat the germicidal, it is necessary to inoculate a second series of tubes from the first so as to decrease still further the amount of antiseptic carried over. The results obtained are signified as follows:  $x$  per cent. of the disinfectant in watery solution and at  $y$  temperature kills the organism in twenty minutes,  $z$  per cent. at the same temperature kills in one minute, and so on. If there be any doubt whether the trace of the disinfectant carried over with the platinum loops may have rendered the gelatin unsuitable for growth, thus falsifying results, control cultures, if extreme accuracy is desired, should be made by adding bacteria which have been somewhat enfeebled by slight contact with the disinfectant to fluid to which a similar trace of the disinfectant has been added. If the strength of the disinfectant is to be tested for different substances it must be tested in these substances or their equivalent, and not in water.

The disinfectant to be examined should always be dissolved in an inert fluid, such as water; if on account of its being insoluble in water it is necessary to use alcohol for its solution, control experiments may be required to determine the action of the alcohol on the organism. Sometimes, as in the case of corrosive sublimate, the chemical unites with the cell substance to form an unstable compound, which inhibits the growth of the organism for a time before destroying it. If this compound is not broken up in the media it will probably not be in the body. In some tests it is of interest to break up this union and note then whether the organism is alive or dead. With corrosive sublimate the bacteria probably die within thirty minutes after the union occurs.

In the above determinations the absolute strength of the disinfectant required is considerably less when culture media poor in albumin are employed than when the opposite is the case. Cholera spirilla grown in bouillon containing no peptone or only 0.5 per cent. of peptone are destroyed in half an hour by 0.1 per cent. of hydrochloric acid; grown in 2 per cent. peptone bouillon, their vitality is destroyed in the same time on the addition of 0.4 per cent. HCl. In any case the organisms to be tested should all be treated in exactly the same way and the results accompanied by a statement of the conditions under which the tests were made. It is becoming the custom to state the power of a disinfectant in terms of comparison with pure carbolic acid. A substance which had the same destructive power in a 1 to 1000 solution as carbolic acid in a 1 to 100 solution would be rated as of a strength ten times that of carbolic acid.

The following table gives the results and methods used in an actual experiment to test the effect of blood serum upon the disinfecting action of bichloride of mercury and carbolic acid upon bacteria:

TEST FOR THE DIFFERENCE OF EFFECT OF BICHLORIDE OF MERCURY AND CARBOLIC ACID SOLUTION ON TYPHOID BACILLI IN SERUM AND IN BOUILLON.

Time.	1'	3'	5'	10'	20'	30'	45'	1 hr.	1½ hrs.	2 hrs.	Strength of solution.
A. Serum . . . 2.5 c.c. HgCl <sub>2</sub> sol. 1:1000 2.5 c.c. Typhoid broth culture. }	+	+	+	-	-	-	-	-	-	-	{ Equals 1:2000 bi- chloride.
B. Bouillon . . . 2.5 c.c. HgCl <sub>2</sub> sol. 1:1000 2.5 c.c. Typhoid broth culture. }	-	-	-	-	-	-	-	-	-	-	
C. Serum . . . 2.5 c.c. Carbolic sol. 5% 2.5 c.c. Typhoid broth culture. }	+	+	-	-	-	-	-	-	-	-	{ Equals 2½% car- bolic acid.
D. Bouillon . . . 2.5 c.c. Carbolic sol. 5% 2.5 c.c. Typhoid broth culture. }	+	-	-	-	-	-	-	-	-	-	

- Indicates total destruction of bacteria with no growth in media.  
+ Indicates lack of destruction of bacteria with growth in media.

THE STANDARDIZATION OF DISINFECTANTS.

Rideal and Walker were the first to urge a useful method for standardizing disinfectants.

In carrying out the test the various factors must be carefully controlled, thus: *Time*: this should be constant, the strength of the disinfectant being the variant. *Test organisms*: a standard culture of the typhoid bacillus (Hopkins' strain) is used to avoid any variations due to the different degrees of resistance of various strains. The culture should be subinoculated three days before used. *Medium*: a standard

meat-extract broth 1.5 per cent. acid to phenolphthalein; 10 c.c. to a tube is employed. *Temperature:* this test is done at 20° C. This is important, as the germicidal activity increases with the temperature. *Constant amount of culture used:* 0.1 c.c. of the twenty-four-hour broth culture is added to 5 c.c. of the disinfectant solution. This is more accurate than the drop method. *Amount inoculated:* it is essential that the same amount be inoculated from each dilution. Platinum loops made of 23 United States gauge wire, the loops being 4 mm. in diameter are employed. Several are used, being left on a rack after sterilization so as to be cold when needed. The loop is bent at an angle of 45° to the shank.

The actual test is carried out as follows: A 5 per cent. carbolic solution (phenol C. P.), is prepared and standardized by bromine titration. From this freshly prepared 1 to 90 to 1 to 100 and 1 to 110 dilutions are made as needed. The necessary dilutions of germicide are then prepared. Wide jumps in the dilutions are made and then narrowed as the limits of the disinfectant to be tested are determined.

Five test-tubes are arranged in a row in a water-bath at 20° C., and the solutions added in 5 c.c. amounts. Time must be allowed for the solution to reach 20° C. If the bath be large enough, little attention is needed to keep the temperature constant. The culture having been brought to 20° C. is then added in 0.1 c.c. amounts and the tubes shaken, an interval of thirty seconds allowed between each tube. Subinoculation of the first tube is then made after thirty seconds, which gives an interval of two and a half minutes after inoculation. The tubes are then subinoculated in order at thirty-second intervals, giving an interval for each of two and one-half minutes after inoculation, and starting at the first, gives an interval of five minutes, etc.

It is possible to use ten tubes, as each step can be done in less than fifteen seconds if properly arranged, allowing a much wider range for each test. It is not necessary to keep the cotton plugs in the tubes during the operation nor to remove the tubes from the bath to obtain the loopful for inoculation. The loop is plunged to the bottom, care being taken not to touch the sides of the tubes, and care should also be taken that a loopful is carried away each time. The broth tubes are incubated for forty-eight hours and then examined for growth.

The following are given as two examples:

Sample.	Dilution.	Time of exposure:						Phenol coefficient.
		2.5 min.	5 min.	7.5 min.	10 min.	12.5 min.	15 min.	
Phenol . . . .	1 to 90	+	—	—	—	—	—	100)550
	1 to 100	+	+	+	—	—	—	5.5
Disinfectant A. . . .	1 to 450	+	+	—	—	—	—	
	1 to 550	+	+	+	—	—	—	
	1 to 600	+	+	+	+	—	—	
Phenol . . . .	1 to 100	+	+	+	—	—	—	110)650
	1 to 110	+	+	+	+	—	—	5.191
Disinfectant A. . . .	1 to 600	+	+	+	+	—	—	
	1 to 650	+	+	+	+	+	—	
	1 to 700	+	+	+	+	+	+	

These tables not only serve as an example, but also show that unless many repetitions of the tests are made and averaged, considerable variations in the results may be obtained. A report of 5.5 or 5.1 is equally accurate in the test here reported of the Rideal-Walker method, even with certain improvements added. With practice, and by selection of the dilutions to be employed, the operator evidently can regulate the time factor so that fairly uniform results are obtainable. On the other hand, it not infrequently happens that if more than one carbolic dilution is employed, more than one time period is open to comparison. For these reasons Anderson and McClintic have modified the test by setting two time limits two and one-half and fifteen minutes and taking the average. The following is an example:

Sample.	Dilution.	Time of exposure:						Phenol coefficient.	
		2.5 min.	5 min.	7.5 min.	10 min.	12.5 min.	15 min.		
Phenol . . .	1 to 80	—	—	—	—	—	—	375	650
	1 to 90	+	—	—	—	—	—		
	1 to 100	+	+	+	—	—	—	80	110
	1 to 110	+	+	+	+	+	—		
Disinfectant A. .	1 to 350	—	—	—					
	1 to 375	—	—	—				4.69 + 5.91	
	1 to 400	+	—	—	—				
	1 to 425	+	+	—	—	—	—		
	1 to 450	+	+	—	—	—	—	2	=
	1 to 500	+	+	—	—	—	—		
	1 to 550	+	+	+	—	—	—	5.30	
	1 to 600	+	+	+	+	—	—		
	1 to 650	+	+	+	+	+	—		
	1 to 700	+	+	+	+	+	+		
	1 to 750	+	+	+	+	+	+		

Disinfectants vary widely in their germicidal properties, depending on whether organic matter is present or not. As under practical conditions organic matter is usually present, it is of some importance to know how far organic matter decreases the efficiency.

For the purpose of obtaining comparable results, Anderson and McClintic have suggested the use of peptone 10 per cent. and gelatin 5 per cent. in distilled water. One part of the culture is mixed with 10 parts of the organic solution, 1.1 c.c. being then added to a series of dilution tubes containing 4 c.c. In determining the coefficient allowance must be made for the added amount of organic matter.

The modified methods of Anderson and McClintic<sup>1</sup> are called the "hygienic laboratory phenol coefficient," with or without organic matter. Any organic matter may be used in the test to approach the special conditions under which a disinfectant is to be used.

In comparing the value of disinfectants the cost as well as the coefficient must be considered. This is best stated in terms of the

<sup>1</sup> See Hygienic Laboratory Bulletin No. 82, for further details and apparatus for simplifying the steps of the test.

relative cost of 100 units of efficiency as compared with pure phenol = 100, thus:

$$\frac{\text{Cost of disinfectant per gallon}}{\text{Cost of phenol per gallon}} \quad (= \text{Cost ratio}) \div \frac{\text{Coefficient of disinfectant.}}{\text{Coefficient of phenol (1).}}$$

( = the efficiency),  $\times 100$  = relative cost per 100 units.

**Antiseptic Value.**—With certain disinfectants there is sufficient of the disinfectant carried over by the loop to exert antiseptic action and growth does not occur. If this is not taken into consideration a disinfectant will be given an excessively high coefficient. No satisfactory method has been devised to avoid this difficulty. The inoculated broth tube may be shaken and a loop or more inoculated from it to second broth tube, in this way diluting the disinfectant still further.

Chick<sup>1</sup> has attempted to overcome the difficulty in the case of mercury-containing disinfectants by adding 0.2 c.c. of a saturated watery solution of hydrogen sulphide to each tube of broth.

Many substances which are strong disinfectants become altered under the conditions in which they are used, so that they lose a portion or all of their germicidal properties; thus, quicklime and milk of lime act by means of their alkali and are disinfecting agents only so long as sufficient calcium hydroxide is present. If this is changed by the carbon dioxide of the air into carbonate of lime it becomes harmless. Bichloride of mercury and many other chemicals form compounds with many organic and inorganic substances, which, though still germicidal, are much less so than the original substances. Solutions of chlorine, peroxides, etc., when in contact with an excess of organic matter soon become inert because of the chemical compounds formed.

**The Disinfecting Properties of Inorganic Compounds.**—**Bichloride of Mercury.**—This substance, which dissolves in 16 parts of cold water, when present in 1 part in 100,000 in nutrient gelatin or bouillon, inhibits the development of most forms of bacteria. In water 1 part in 50,000 will kill many varieties in a few minutes, but in bouillon twenty-four hours may be needed. With organic substances its power is lessened, so that 1 part to 1000 may be required. Most spores are killed in 1 to 500 watery solution within one hour. Corrosive sublimate is less effective as a germicide in alkaline fluids containing much albuminous substance than in watery solution. In such fluids, besides loss in other ways, precipitates of albuminate of mercury are formed which are at first insoluble, so that a part of the mercuric salt does not really exert any action. In alkaline solutions, such as blood, blood serum, pus, sputum, tissue fluids, etc., the soluble compounds of mercury are converted into oxides or hydroxides.

For ordinary use, where corrosive sublimate is employed, solutions of 1 to 500 and 1 to 2000 will suffice, when brought in contact with bacteria, to kill the vegetative forms within from one to twenty minutes, the stronger solution to be used when much organic matter is present.

<sup>1</sup> Journal of Hygiene, 1908, viii, 654.

Mercuric chloride volatilizes slowly and it is better to wash off walls after use of bichloride solutions. Solutions of this salt should not be kept in metal receptacles. Mercuric chloride solution has disadvantages in that it corrodes metals, irritates the skin, and forms almost inert compounds with albuminous matter. In order to avoid accidents, solutions of this odorless disinfectant should be colored by some dye.

**Biniiodide of Mercury.**—This salt is very similar in its effect to the bichloride.

**Nitrate of Silver.**—Nitrate of silver in watery solution has about one-fourth the value of the bichloride of mercury as a disinfectant, but nearly the same value in inhibiting growth. In albuminous solutions it is equal to bichloride of mercury. Compounds of silver nitrate and albuminous substances have been used because of the absence of irritating properties combined with moderate antiseptic power.

**Sulphate of Copper.**—This salt has about 50 per cent. of the value of mercuric chloride. It has a quite remarkable affinity for many species of algae, so that when in water 1 to 1,000,000 it destroys many forms; 1 to 400,000 destroys typhoid bacilli in twenty-four hours when the water has no excessive amount of organic material. It is not known to be poisonous in this strength, so that it can be temporarily added to water supplies.

**Sulphate of Iron.**—This is a much less powerful disinfectant than sulphate of copper. A 5 per cent. solution requires several days to kill the typhoid bacilli. It can only be considered as a mild antiseptic and deodorant.

**Zinc Chloride.**—This is very soluble in water, but is a still weaker disinfectant than copper sulphate.

**Sodium Compounds.**—A 30 per cent. solution of NaOH kills anthrax spores in about ten minutes, and in 4 per cent. in about forty-five minutes. One per cent. kills vegetative forms in a few minutes. Sodium carbonate kills spores with difficulty even in concentrated solution, but at 85° C. it kills spores in from eight to ten minutes. It is used frequently to cover metallic instruments. A 5 per cent. solution kills in a short time the vegetative forms of bacteria. Even ordinary soapsuds have a slight bactericidal as well as a marked cleansing effect. The bicarbonate has almost no destructive effect on bacteria.

**Calcium Compounds.**—Calcium hydroxide,  $\text{Ca(OH)}_2$ , is a powerful disinfectant; the carbonate, on the other hand, is almost without effect. The former is prepared by adding one pint of water to two pounds of lime (quicklime,  $\text{CaO}$ ). Exposed to the air the calcium hydrate slowly becomes the inert carbonate. A 1 per cent. watery solution of the hydroxide kills bacteria which are not in the spore form within a few hours. A 3 per cent. solution kills typhoid bacilli in one hour. A 20 per cent. solution added to equal parts of feces or other filth and mixed with them will completely sterilize them within two hours.

**Effect of Acids.**—An amount of acid per liter which is equivalent to 40 c.c. of normal hydrochloric acid is sufficient to prevent the growth of all

varieties of bacteria and to kill many. Twice this amount destroys most bacteria within a short time. The variety of acid makes little difference. Bulk for bulk, the mineral acids are more germicidal than the vegetable acids, but that is because their molecular weight is so much less. A 1 to 500 solution of sulphuric acid kills typhoid bacilli within one hour. A similar solution of hydrochloric acid is about one-third weaker, and acetic acid somewhat weaker still. Citric, tartaric, malic, formic, and salicylic acids are similar to acetic acid. Boric acid destroys the less resistant bacteria in 2 per cent. solution and inhibits the others.

**Gaseous Disinfectants.**—The germicidal action of gases is much more active in the presence of moisture than in a dry condition.

**Sulphur Dioxide ( $\text{SO}_2$ ).**—Numerous experiments have been made with this gas owing to the fact that it has been so extensively used for the disinfection of hospitals, ships, apartments, clothing, etc. This gas is a much more active germicide in a moist than in a dry condition; due, no doubt, to the formation of the more active disinfecting agent—sulphurous acid ( $\text{H}_2\text{SO}_3$ ). In a pure state anhydrous sulphur dioxide does not destroy spores, and is not certain to destroy bacteria in the vegetative form. Sternberg has shown that the spores of the *Bacillus anthracis* and *Bacillus subtilis* are not killed by contact for some time with liquid  $\text{SO}_2$  (liquefied by pressure). Koch found that various species of spore-bearing bacilli exposed for ninety-six hours in a disinfecting chamber to the action of  $\text{SO}_2$ , in the proportion of from 4 to 6 per cent. by volume, were not destroyed. In the absence of spores, however, the anthrax bacillus in a moist condition, attached to silk threads, was found by Sternberg to be destroyed in thirty minutes in an atmosphere containing 1 per cent. by volume.

As the result of a large number of experiments with  $\text{SO}_2$  as a disinfectant it has been determined that an "exposure for eight hours to an atmosphere containing at least 4 volumes per cent. of this gas *in the presence of moisture*" will destroy most, if not all, of the pathogenic bacteria in the absence of spores. Four pounds of sulphur burned for each 1000 cubic feet will give an excess of gas.

**Peroxide of Hydrogen ( $\text{H}_2\text{O}_2$ ).**—This is an energetic disinfectant, and in 2 per cent. solution (about 40 per cent. of the ordinary commercial article) will kill the spores of anthrax in from two to three hours. A 20 per cent. solution of a good commercial hydrogen peroxide solution will quickly destroy the pyogenic cocci and other spore-free bacteria. It combines with organic matter, becoming inert. It is prompt in its action and not poisonous, but apt to deteriorate if not properly kept.

**Chlorine.**—Chlorine is a powerful gaseous germicide, owing its activity to its affinity for hydrogen and the consequent release of nascent oxygen when it comes in contact with microorganisms in moist condition. It is therefore a much more active germicide in the presence of moisture than in a dry condition. Thus, Fischer and Proskauer found that dried anthrax exposed for an hour in an atmosphere containing 44.7



per cent. of dry chlorine were not destroyed; but if the spores were previously moistened and were exposed in a moist atmosphere for the same time, 4 per cent. was effective, and when the time was extended to three hours 1 per cent. destroyed their vitality. The anthrax bacillus, in the absence of spores, was killed by exposure in a moist atmosphere containing 1 part to 2500 for twenty-four hours.

In watery solutions 0.2 per cent. kills spores within five minutes and the vegetative forms almost immediately. One part in one million is sufficient to destroy typhoid bacilli, in a water containing little organic matter, in the course of a few hours. In water with much organic matter a much greater amount is required.

**Chlorinated Lime (Called "Chloride of Lime").**—Chlorinated lime is made by passing nascent chlorine gas over unslaked lime. It should not contain less than 10 per cent. of available chlorine, and can now be obtained containing 30 per cent. It should have a strong odor of chlorine. Its efficacy depends on the chlorine it contains in the form of hypochlorites. The calcium hypochlorite is readily broken up into hypochlorous acid. A solution in water of 0.5 to 1 per cent. of chlorinated lime will kill most bacteria in one to five minutes, and 1 part in 100,000 will destroy typhoid bacilli in twenty-four hours. A 5 per cent. solution usually destroys spores within one hour. Chlorinated lime not only bleaches, but destroys fabrics.

**The Hypochlorites (Labarraque's Solution).**—Solutions of hypochlorites are practically the same as solutions of chlorinated lime and are much more expensive.

**Bromine and iodine** are of about the same value as chlorine for gaseous disinfectants, in the moist condition; but, like chlorine, they are not applicable for general use in house disinfection, owing to their poisonous and destructive properties; they have a use in sewers and similar places.

**Trichloride of iodine** in 0.5 per cent. solution destroys the vegetative forms of bacteria in five minutes.

**Organic Disinfectants.**—**Alcohol** in 10 per cent. solution inhibits the growth of bacteria; absolute alcohol kills bacteria in the vegetative form in from several to twenty-four hours. According to Epstein, 50 per cent. alcohol (in water) has more germicidal power than any other strength, the power gradually diminishing with both stronger and weaker solutions.

**Formaldehyde.**—Formaldehyde, or formic aldehyde, was isolated by von Hoffmann in 1867, who obtained it by passing the vapors of methyl alcohol mixed with air over finely divided platinum heated to redness. The methyl alcohol is oxidized and produces formaldehyde as follows:



Formaldehyde is a gaseous compound possessed of an extremely irritating odor. At a temperature of 68° F. the gas is polymerized—that is to say, a second body is formed, composed of a union of two

molecules of  $\text{CH}_2\text{O}$ . This is known as a paraformaldehyde, and is a white, soapy body, soluble in boiling water and in alcohol. Formaldehyde is sold in commerce as a clear, watery liquid containing from 33 to 40 per cent. of the gas and 10 to 20 per cent. of methyl alcohol, its chief impurity. If the commercial solution—ordinarily known in the trade as “formalin”—is evaporated or concentrated above 40 per cent., paraformaldehyde results; and when this is dried *in vacuo* over sulphuric acid a third body—trioxymethylene—is produced, consisting of three molecules of  $\text{CH}_2\text{O}$ . This is a white powder, almost insoluble in water or alcohol, and giving off a strong odor of formaldehyde. The solid polymers of formaldehyde, when heated, are again reduced to an active gaseous condition; ignited, they finally take fire and burn with a blue flame, leaving but little ash. When burned they have no germicidal properties.

Formaldehyde has an active affinity for many organic substances, and forms with some of them definite chemical combinations. It combines readily with ammonia to produce a compound called hexamethylene tetramine, which possesses neither the odor nor the antiseptic properties of formaldehyde. This action is made use of in neutralizing the odor of formaldehyde when it is desired to dispel it rapidly after disinfection.

Formaldehyde also forms combinations with certain aniline colors—viz., fuchsin and safranin—the shades of which are thereby changed or intensified. These dyes are tests for aldehydes. These are the only colors, however, which are thus affected, and as they are seldom used in dyeing, owing to their liability to fade, this effect is of little practical significance. The most delicate fabrics of silk, wool, cotton, fur, etc., are unaffected in texture or color by formaldehyde. Iron and steel are attacked, after long exposure, by the gas in combination with watery vapor; but copper, brass, nickel, zinc, silver, and gilt work were not at all acted upon.

Formaldehyde unites with nitrogenous products of decay—fermentation or decomposition—forming true chemical compounds, which are odorless and sterile. It is thus a true deodorizer in that it does not replace one odor by another more powerful, but forms new chemical compounds which are odorless. Formaldehyde has a peculiar action upon albumin, which it transforms into an insoluble and indecomposable substance. It renders gelatin insoluble in boiling water and most acids and alkalies. It is from the property of combining chemically with the albuminoids forming the protoplasm of bacteria that formaldehyde is supposed to derive its bactericidal powers. Formaldehyde is an excellent preservative of organic products. It has been used for the preservation of meat, milk, and other food products; but, according to Trillat and other investigators, formaldehyde renders these substances indigestible and unfit for food. It has been successfully employed as a preservative of pathological and histological specimens.

There are no exact experiments recorded of the physiological action of formaldehyde on the human subject when taken internally. A 1 per cent. solution has been taken in considerable quantity without

which *salmonella* and *shigella* have been given in doses up to 100 grains as an intestinal antiseptic. According to Aronson rabbits and guinea-pigs allowed to remain for twelve and twenty-four hours in rooms which were being disinfected with formaldehyde gas were found to be perfectly well when the rooms were opened. On autopsy the animals showed no injurious effects of the gas. Others have noticed that animals, such as dogs and cats, which have accidentally been confined for any length of time in rooms undergoing formaldehyde disinfection occasionally died from the effects of the gas. Many observers, however, have reported that insects, such as roaches, flies, and bedbugs, are not, as a rule, affected. The result of these observations would seem to indicate that although formaldehyde is comparatively non-toxic to the higher forms of animal life, nevertheless a certain degree of caution should be observed in the use of this agent. It is important to remember that formaldehyde in gaseous form is practically inert as an insecticide except in extremely great concentrations.

The researches of Pottevin and Trillat have shown that the germicidal power of the gas depends not only upon its concentration, but also upon the temperature and the condition of the objects to be sterilized. As with other gaseous disinfectants—viz., sulphur dioxide and chlorine—it has been found that the action is more rapid and complete at high temperatures—i. e., at 35° to 45° C. (95° to 120° F.)—and when the test objects are moist than at lower temperatures and when the objects are dry. Still, it has been repeatedly demonstrated by actual experiment in rooms that it is possible to disinfect the surface of apartments and articles contained in them, under the conditions of temperature and moisture ordinarily existing in rooms even in winter, by an exposure of a few hours to a saturated atmosphere of formaldehyde gas. The results of numerous experiments have shown that in the air 2.5 per cent. by volume of the aqueous solution, or 1 per cent. by volume of the gas, are sufficient to destroy fresh virulent cultures of the common non-spore-bearing pathogenic bacteria in a few minutes.

Stahl has shown that bandages and iodoform gauze can be kept well sterilized by placing in the jars containing pieces of a preparation of paraformaldehyde in tablet form containing 50 per cent. of formaldehyde. The same experimenter has also succeeded in making carpets and articles of clothing germ-free by spraying them with 0.5 to 2 per cent. solution of formaldehyde for fifteen to twenty minutes without the color of the fabrics being in any way affected. The investigations of Trillat, Aronson, Pottevin, and others have shown that a concentration of  $\frac{1}{100}$  of the aqueous solution (40 per cent.), equal to  $\frac{1}{250}$  of pure formaldehyde, was safe and sufficiently powerful to retard bacterial growth.

A 2 per cent. watery solution of formalin destroys the vegetative forms of bacteria within five to thirty minutes. In our experiments formalin has upon the vegetative forms about one-half the strength of carbolic acid.

**Chloroform** ( $\text{CHCl}_3$ ).—This substance, even in pure form, does not destroy spores, although it kills bacteria in vegetative form, even in 1 per cent. solution. Chloroform is used practically as an antiseptic in antitoxic sera and in blood serum for culture purposes. The chloroform is expelled from the serum by heating it to  $55^\circ \text{C}$ .

**Iodoform** ( $\text{CHI}_3$ ).—This substance has but very little destructive action upon bacteria; indeed, upon most varieties it has no appreciable effect whatever. When mixed with putrefying matter, wound discharges, etc., the iodoform is reduced to soluble iodine compounds, which act partly by destroying the bacteria and partly by uniting with poisons already produced.

**Carbolic Acid** ( $\text{C}_6\text{H}_5\text{OH}$ ).—Pure phenol crystallizes in long, colorless crystals. In contact with air it deliquesces. It has a penetrating odor, a burning taste, and is a corrosive poison. It is soluble at ordinary temperatures in about 15 parts of water. Carbolic acid dissolves in water with some difficulty and should be therefore thoroughly mixed. It is not destructive to fabrics, colors, metals, or wood, and does not combine as actively with albuminous matters as bichloride of mercury. It is therefore more suitable for the disinfection of feces, etc. A solution having 1 part to 1000 inhibits the growth of bacteria; 1 part to 400 kills the less resistant bacteria, and 1 part to 100 kills the remainder. A 5 per cent. solution kills the less resistant spores within a few hours and the more resistant in from one day to four weeks. A slight increase in temperature aids the destructive action; thus, even at  $37.5^\circ$  spores are killed in three hours. A 3 per cent. solution kills streptococci, staphylococci, anthrax bacilli, etc., within one minute. Carbolic acid loses much of its value when in solution in alcohol or ether. An addition of 0.5 HCl aids its activity. Carbolic acid is so permanent and so comparatively little influenced by albumin that it is widely used in practical disinfection even in place of more powerful substances.

**Cresol**.—Cresol [ $\text{C}_6\text{H}_4(\text{CH}_3)\text{OH}$ ] is the chief ingredient of the so-called "crude carbolic acid." This is almost insoluble in water, and therefore of restricted value. Many methods are used for bringing it into solution so as to make use of its powerful disinfecting properties. With equal parts of crude sulphuric acid it is a powerful disinfectant, but it is, of course, strongly corrosive. An alkaline emulsion of the cresols and other products contained in "crude" carbolic acid with soap is called creolin. It is used in 1 to 5 per cent. emulsions. It is fully as powerful as pure carbolic acid. Lysol is similar to creolin, except that it has more of the cresols and less of the other products. It and creolin are of about the same value.

**Tricresol**.—Tricresol is a refined mixture of the three cresols (metacresol, paracresol, and orthocresol). It is soluble in water to the extent of 2.5 per cent. and its disinfecting power is about three times as great as that of carbolic acid.

**Creolin**.—Creolin contains 10 per cent. of cresols held in solution by soap.

**Lysol.**—Lysol contains about 50 per cent. of cresols. It mixes with water in all dilutions.

**Oil of Turpentine**, 1 to 200, prevents the growth of bacteria.

**Camphor** has very slight antiseptic action.

**Creosote** in 1 to 200 kills many bacteria in ten minutes; 1 to 100 failed to kill tubercle bacilli in twelve hours.

**Essential Oils.**—Cardéac and Meumir found that the essences of cinnamon, cloves, thyme, and others killed typhoid bacilli within one hour. Sandalwood required twelve hours.

Thymol and eucalyptol have about one-fourth the strength of carbolic acid (Behring).

Oil of peppermint in 1 to 100 solution prevents the growth of bacteria.

TABLES OF ANTISEPTIC VALUES.<sup>1</sup>

Alum . . . . .	1 to 222	Mercuric chloride . . . . .	1 to 14,300
Aluminum acetate . . . . .	1 to 6000	Mercuric iodide . . . . .	1 to 40,000
Ammonium chloride . . . . .	1 to 9	Potassium bromide . . . . .	1 to 10
Boric acid . . . . .	1 to 143	Potassium iodide . . . . .	1 to 10
Calcium chloride . . . . .	1 to 25	Potassium permanganate . . . . .	1 to 300
Calcium hypochlorite . . . . .	1 to 1000	Pure formaldehyde . . . . .	1 to 25,000
Carbolic acid . . . . .	1 to 333	Quinine sulphate . . . . .	1 to 800
Chloral hydrate . . . . .	1 to 107	Silver nitrate . . . . .	1 to 12,500
Cupric sulphate . . . . .	1 to 2000	Sodium borate . . . . .	1 to 14
Ferrous sulphate . . . . .	1 to 200	Sodium chloride . . . . .	1 to 6
Formaldehyde (40%) . . . . .	1 to 10,000	Zinc chloride . . . . .	1 to 500
Hydrogen peroxide . . . . .	1 to 20,000	Zinc sulphate . . . . .	1 to 20

<sup>1</sup> These figures are approximately correct, and represent the percentage of disinfection required to be added to a fluid containing considerable organic material, in order permanently to prevent any bacterial growth. Solutions of half the given strength will inhibit the growth of most bacteria and prevent the growth of many varieties.

AVERAGE ACTION OF REPRESENTATIVE DISINFECTANTS ON SOME OF THE MORE IMPORTANT PATHOGENIC BACTERIA.

Kind of bacteria.	Thermal death-point.			Kind of media.	Mercuric chloride, 1 to 1000.	Hydrogen peroxide, 1 to 200.	Carbolic acid, 1 to 100.	Formalin, 1 to 50.	Trichloride of iodine, 1 to 750.	Copper sulphate, 1 to 200.	Tricresol, 1 to 200.	Chloramin, 1 to 10,000.
	100°	80°	55° C.									
Typhoid bacilli . . .	..	..	15 <sup>1</sup>	{ Non-albuminous Albuminous <sup>2</sup>	1 5	5 20	5 15	5 15	..	..	1 5	..
B. coli . . . . .	..	..	20	{ Non-albuminous Albuminous	5 70	..	15 70	20	..	..	..	..
Diphtheria bacillus . .	..	5	20	{ Non-albuminous Albuminous	1 5	10 20	5 10	3 5	1 5	2 5	1 5	..
Staphylococcus . . .	2	5	60	{ Non-albuminous Albuminous	15 30	15 30	20 30	20 40	..	..	..	..
Streptococcus . . . .	..	..	20	{ Non-albuminous Albuminous	5 10	5 15	5 40	5 30	5 ..	5 10	5 10	..
Pneumococcus . . . .	..	..	20	{ Non-albuminous Albuminous	5 ..	5 ..	5 10	5 10	5 ..	5 ..	5 ..	..
Meningococcus . . . .	..	..	25	{ Non-albuminous Albuminous	1 5	3 15	1 5	1 5	..	..	1 5	..
A. anthracis spores .	150	..	..	{ Non-albuminous Albuminous	180 at 75° C.	..	150 at 75° C.	..	..	..	..	..

<sup>1</sup> The time is given in minutes.<sup>2</sup> The tests made with albuminous media were by Willa Noble in our Laboratory. The cultures were grown twenty-four hours at 37° C. in glucose ascitic broth, one-tenth of culture inoculated into 5 c.c. of disinfectant, temperature 20° C.

## CHAPTER LIII.

### PRACTICAL DISINFECTION AND STERILIZATION (HOUSE, PERSON, INSTRUMENTS, AND FOOD). STERILIZATION OF MILK FOR FEEDING INFANTS.

#### DISINFECTANTS AND METHODS OF DISINFECTION EMPLOYED IN THE HOUSE AND SICK-ROOM.

**Disinfection and Disinfectants.**—Sunlight, pure air, and cleanliness are always very important agents in maintaining health and in protecting the body against many forms of illness. When, however, it becomes necessary to guard against such special dangers as infectious material from communicable diseases the additional protection of disinfection should be considered. Practical disinfection never affords complete protection; and perfect cleanliness is better, even in the presence of contagious disease, than filth with disinfection, as it is ordinarily carried out. If it is possible to disinfect thoroughly the discharge of patients and of those in contact with them aërial disinfection is unnecessary.

In order that as few articles as possible shall be exposed to the pathogenic germs in the discharges of patients, it is important when conditions allow of it that all articles not necessary for the care and comfort of the sick person, especially upholstered furniture, carpets, and curtains, should be removed from the room before placing the sick person in it.

**Agents for Cleansing and Disinfection.**—Too much emphasis cannot be placed upon the importance of cleanliness, both as regards the person and the dwelling, in protecting the body from all kinds of infectious disease. Personal cleanliness should be attained by frequently washing the hands and body, replacing fabrics infected by expectoration, bowel discharges, etc. By these means most of the pathogenic bacteria are removed before they have caused infection.

Cleanliness in dwellings, and in all places where men go, may, under ordinary circumstances, be well maintained by the use of the two following solutions:

1. **Soapsuds Solution.**—For simple cleansing, or for cleansing after the method of disinfection by chemicals described below, one ounce of common washing soda should be added to twelve quarts of hot soapsuds (soft soap and water).

2. **Strong Soda Solution.**—This, which is a stronger and more effective cleansing solution and also a fairly efficient disinfectant, is made by dissolving one-half pound of common washing soda in three gallons of water. The solution thus obtained should be applied by scrubbing with a hard brush.

When it becomes necessary to prevent the spread of communicable diseases by surely killing the living germs which cause them, more powerful agents must be employed than those required for simple cleanliness, and these are commonly called disinfectants. The following are some of the most reliable ones:

1. **Heat.**—Complete destruction by fire is an absolutely safe method of disposing of infected articles of small value, but continued high temperatures not as great as that of fire will destroy all forms of life; thus, boiling or steaming in closed vessels for ten minutes will absolutely destroy all disease germs except spores.

2. **Carbolic Acid Solution.**—Dissolve six ounces of carbolic acid in one gallon of hot water (200 grams in 4000 c.c.). This makes approximately a 5 per cent. solution of carbolic acid, which, for most purposes, may be diluted with an equal quantity of water. The commercial "soluble crude carbolic acid" which is cheaper and twice as effective as the carbolic acid, can be used for privies and drains.<sup>1</sup> It makes a white emulsion on account of its not entering readily into solution. Care must be taken that the pure acid does not come in contact with the skin.

3. **Bichloride Solution** (bichloride of mercury or corrosive sublimate).—Dissolve sixty grains of pulverized corrosive sublimate and two tablespoonfuls of common salt in one gallon of hot water. This solution, which is approximately 1 to 1000, must be kept in glass, earthen, or wooden vessels (not in metal vessels). For safety it is well to color the solution.

The carbolic and bichloride solutions are very poisonous when taken by the mouth, but are harmless when used externally.

4. **Milk of Lime.**—This mixture is made by adding one quart of dry, freshly slaked lime to four or five quarts of water. (Lime is slaked by pouring a small quantity of water on a lump of quicklime. The lime becomes hot, crumbles, and as the slaking is completed a white powder results. The powder is used to make milk of lime.) Air-slaked lime (the carbonate) has no value as a disinfectant.

5. **Dry Chlorinated Lime, "Chloride of Lime."**—This must be fresh and kept in closed vessels or packages. It should have the strong, pungent odor of chlorine (see page 664).

6. **Formalin** (this is a watery solution containing 40 per cent. of formaldehyde).—Add 1 part of formalin to 10 of water. This equals in value the 5 per cent. carbolic acid solution.

7. **Creolin, Tricresol, and Lysol.**—The first is of about the same value as pure carbolic acid, the latter two about three times as powerful.

The proprietary disinfectants, which are so often widely advertised and whose composition is kept secret, are relatively expensive and often

<sup>1</sup> The cost of the pure carbolic acid solution is much greater than that of most of the other solutions, but except for the disinfection of the skin, which in some persons it irritates, and of woodwork, it is generally much to be preferred by those not thoroughly familiar with disinfectants, as it does not deteriorate, and is rather more uniform in its action than some of the other disinfectants.



unreliable and inefficient. It is important to remember that substances which destroy or disguise bad odors are not necessarily disinfectants, and that there are very few disinfectants that are not poisonous when taken internally. Their value should be stated in the circular in comparison with pure carbolic acid, so that their strength may be known.

**Methods of Disinfection in Infectious and Contagious Diseases.—**

The diseases most commonly guarded against, outside of surgery, by disinfection are scarlet fever, measles, diphtheria, tuberculosis, small-pox, typhoid, bubonic plague, and cholera.

1. **Hands and Person.**—Dilute the 5 per cent. carbolic solution with an equal amount of water or use the 1 to 1000 bichloride solution without dilution. Hands soiled in caring for persons suffering from contagious diseases, or soiled portions of the patient's body, should be immediately and thoroughly soaked with one of these solutions and then washed with soap and water, and finally immersed again in the solution. The nails should always be kept clean. Before eating, the hands should be first washed in one of the above solutions, and then thoroughly scrubbed with soap and water by means of a brush.

2. **Soiled clothing, towels, napkins, bedding, etc.,** should on removal be immediately immersed in the 2.5 per cent. carbolic solution, in the sick room, and soaked for one or more hours. Articles such as beds, woollen clothing, etc., which cannot be washed, should be thoroughly exposed to formaldehyde gas, as noted later (see page 674). This is not necessary for goods after exposure to measles.

3. **Food and Drink.**—Food thoroughly cooked and drinks that have been boiled are free from disease germs. Food and drinks, after cooking or boiling, if not immediately used, should be placed when cool in clean dishes or vessels and covered. In the presence of an epidemic of cholera or typhoid fever, milk and water used for drinking, cooking, washing dishes, etc., should be boiled before using, and all persons should avoid eating uncooked fruit and fresh vegetables.

4. **Discharges of all kinds from the mouth, nose, bladder, and bowels** of patients suffering from contagious diseases should be received into glass, metal, or earthen vessels containing the carbolic solution, or milk of lime, or they should be removed on pieces of cloth, which are immediately immersed in one of these solutions or boiled or destroyed by fire. Special care should be observed to disinfect at once the vomited matter and the intestinal discharges from cholera patients. In typhoid fever the urine and the intestinal discharges, and in diphtheria, measles, and scarlet fever the discharges from the throat and nose all carry infection and should be treated in the same manner. The volume of the solution used to disinfect discharges should be at least twice as great as that of the discharge, and should completely mix with it and cover it. After standing for an hour or more the discharges with the exception of the feces may be thrown into the water-closet.

Masses of feces are extremely difficult to disinfect except on the surface, for it takes disinfectants such as the carbolic acid solution

some twelve hours to penetrate to their interior. If fecal masses are to be thrown into places where the disinfectant solution covering them will be washed off, it will be necessary to be certain that the disinfectant has previously penetrated to all portions and destroyed the disease germs. This can be brought about by stirring them with the disinfectant and allowing the mixture to stand for one hour, or by washing them into a pot holding soda solution which is already at the boiling temperature, or later will be brought to it.

**5. Sputum from Consumptives.**—The importance of the proper disinfection of the sputum is still underestimated. Consumption is an infectious disease, and is always the result of transmission from the sick to the healthy or from animals to man. The sputum contains the germs which cause the disease, and in a large proportion of cases is the source of infection. After being discharged, unless properly disposed of, it may become dry and pulverized and float in the air as dust. This dust contains the germs, and is a common cause of the disease, through inhalation. In all cases, therefore, the sputum should be disinfected when discharged. It should be received in covered cups containing the carbolic or milk-of-lime solution. Handkerchiefs soiled by it should be soaked in the carbolic solution and then boiled. Dust from the walls, mouldings, pictures, etc., in rooms that have been occupied by consumptive patients, where the rules of cleanliness have not been carried out, contain the germs and will produce tuberculosis in animals when used for their inoculation; therefore rooms should be thoroughly renovated or disinfected before they are again occupied. If the sputum of all consumptive patients were destroyed at once when discharged a large proportion of the cases of the disease would be prevented.

**6. Closets, Kitchen and Hallway Sinks, etc.**—The closet should never be used for infected discharges until they have been thoroughly disinfected; when done, one quart of carbolic solution or of 5 per cent. solution of formalin should be poured into the pan (after it is emptied) and allowed to remain there. Sinks should be flushed with one of these solutions at least once daily.

**7. Dishes, knives, forks, spoons, etc.,** used by a patient should, as a rule, be kept for his exclusive use and not removed from the room. They should be washed first in the carbolic solution, then in boiling hot soapsuds, and finally rinsed in hot water. The remains of the patient's meals may be burned or thrown into a vessel containing the carbolic solution or milk of lime, and allowed to stand for one hour before being thrown away.

**8. Rooms and Their Contents.**—When the patient is freed from isolation probably the disease germs have already died, but a few may have survived. The danger from infection is much greater when cases are removed during the acute illness. For disinfecting rooms careful fumigation with formaldehyde gas should be employed unless vermin are to be killed, when sulphur fumes should be substituted. Carpets, curtains, and upholstered furniture which have been soiled by discharges, or which have been exposed to infection in the room during the illness, may

be removed for disinfection to chambers where they can be exposed to formaldehyde gas and moderate warmth for twelve to twenty-four hours, or to steam. Some carpets, such as many Wiltons, are discolored by moist steam. These must be put in the formaldehyde chamber. This difficult procedure may be safely omitted, for even if a few germs remain alive in the depth of the fabrics, they probably cannot escape. Wood-work, floors, and plain furniture will be thoroughly washed with soapsuds and bichloride solutions. After the disinfection is finished it is well to remove the dried bichloride of mercury from the walls.

9. **Rags, cloths, and articles of small value**, which have been soiled by discharges or infected in other ways, should be boiled or burned.

10. **In case of death** the body should be completely wrapped in several thicknesses of cloth wrung out of the carbolic or bichloride solution, and when possible placed in an hermetically sealed coffin.

It is important to remember that *an abundance of fresh air, sunlight, and absolute cleanliness* not only helps protect the attendants from infection and aids in the recovery of the sick, but directly eliminates the bacteria which cause disease.

**Methods of Cleanliness and Disinfection to Prevent the Occurrence of Illness.**—1. **Water-closet bowls and all receptacles for human excrement** should be kept perfectly clean by frequent flushing with a large quantity of water, and as often as necessary disinfected with the crude carbolic, or other efficient solutions. The wood-work around and beneath them should be frequently scrubbed with the hot soapsuds solution.

2. **Cesspools and Privy Vaults.**—An abundance of milk of lime or chloride of lime should be thrown into these daily, and their contents should be frequently removed.

3. **The wood-work in school-houses** should be scrubbed daily with hot soapsuds. This refers to floors, doors, door-handles, and all wood-work touched by the scholars' hands.

4. **Spittoons in all public places** should be emptied daily and washed with the hot soapsuds solution, after which a small quantity of the carbolic solution or milk of lime should be put in the vessel to receive the expectoration. This prevents flies conveying infection.

5. **Cars, Ferry-boats, and Public Conveyances.**—The floors, door-handles, railings, and all parts touched by the hands of passengers should be washed frequently with the hot soapsuds solution. Slat-mats from cars, etc., should be cleaned by scrubbing with a stiff brush in the hot soapsuds solution.

Telephone receiver mouth-pieces should also be frequently cleansed.

**The Practical Employment of Formaldehyde Gas in the Surface Disinfection of Rooms and the Disinfection of Goods which would be Injured by Heat.**—Formaldehyde gas has come into such general use, and is for many purposes so valuable, that the description of methods employed to generate and use it will be given in detail.

If we consider now the practical application of formaldehyde gas for purposes of disinfection we find that its destructive action on micro-

organisms depends upon a number of factors, the chief of which are its concentration in the surrounding atmosphere, the length of the contact, the existing temperature, the accompanying moisture, and the nature of the organism. It is not an insecticide like sulphur dioxide.

**Concentration.**—The necessary concentration of gas in the surrounding atmosphere to kill the microorganisms varies with each species, for some resist chemical agents much more than others, and also with the freedom of access of the gas to the bacteria, for if they are under cover or within fabrics a greater amount of gas must be generated than if they are freely exposed.

For purely surface disinfection, when the common pathogenic bacteria are to be destroyed, there will be required, according to the method used, ten to twelve ounces of formalin of full strength, or its equivalent, to 1000 cubic feet of air space.

The gas penetrates through fabrics with difficulty, and to pass through heavy goods the concentration of the gas must be doubled and moderate heat added (45° C. or above).

Although formaldehyde gas does destroy bacteria with the amount of moisture usually present in the air, it acts much more powerfully and certainly when additional moisture is present, and best when present up to the point of saturation. The actual spraying with water of walls and goods to be disinfected is even more efficacious.

**Temperature.**—A fairly high temperature—but one still below that which would injure delicate fabrics—increases not only the activity of formaldehyde gas, but also its penetrative power, and for heavy goods it is essential. The production of a partial vacuum in the chambers before the introduction of the formaldehyde gas still further assists its penetration.

**Length of Exposure.**—This depends upon the nature of the disease for which it is carried out—the penetration required, the concentration of the gas used, the amount of moisture in the air, the temperature of the air, and the size and shape of the room. For surface disinfection in rooms, when as much as twelve ounces of formalin are used for each 1000 cubic feet, five hours' exposure is amply sufficient, most bacteria being killed within the first thirty minutes. For the destruction of microorganisms protected by even a layer of thin covering, double the formalin and double the time of exposure should be allowed, and even then the killing of many species of non-spore-bearing bacteria cannot be counted upon in ordinary rooms. When absolutely complete disinfection is demanded, where penetration of gas is required, the goods must be placed in chambers where moderate heat can be added and all leakage of gas prevented.

In order to insure complete sterilization of the articles they should be so placed as to allow of a free circulation of the gas around them—that is, in the case of bedding, clothing, etc., these should either be spread out on perforated wire shelves or loosely suspended in the chamber. The aid of a partial vacuum facilitates the operation. Upholstered furniture and articles requiring much space should be placed

in a large chamber, or, better, in a room which can be heated to the required temperature.

The most delicate fabrics, furs, leather, and other articles, which are injured by steam, hot air at 230° F., or other disinfectants, are unaffected by formaldehyde.

**DISINFECTION OF BOOKS.**—Books may be satisfactorily disinfected by means of formaldehyde gas in a special room, or in the ordinary steam chamber, as above described, and under the same condition of volume of gas, temperature, and time of exposure. The books should be arranged to stand as widely open as possible upon perforated wire shelves, set about one or one and a half feet apart in the chamber. A chamber having a capacity of 200 to 250 cubic feet would thus afford accommodation for about one hundred books at a time.

Books, with the exception of their surfaces, cannot be satisfactorily disinfected by formaldehyde gas in the bookcases of houses or libraries, or anywhere except in special chambers constructed for the purpose, because the conditions required for their thorough disinfection cannot otherwise be complied with.

**DISINFECTION OF CARRIAGES, ETC.**—Carriages, ambulances, cars, etc., can easily be disinfected by having built a small, tight building, in which they are enclosed and surrounded with formaldehyde gas. Such a building is used for disinfecting ambulances in New York City. With the apparatus there employed a larger amount of formalin is rapidly vaporized, and superficial disinfection is completed in sixty minutes.

**Methods of Generating Formaldehyde Gas.**—Various forms of apparatus can be properly employed to liberate formaldehyde gas for purposes of disinfection. There are two essentials to any good method—namely, that the formaldehyde gas is given off quickly, and that there is no great loss by deterioration of the formalin.

**Wood Alcohol.**—A number of lamps have been devised, all very much on the same principle, though varying somewhat in mechanical construction, which bring about the incomplete oxidation of methyl alcohol by passing the vapors mixed with air over the incandescent metal. Although disinfection can be carried out by the best of these lamps, in our experience none of them up to the present time is satisfactory or economical. They may be very useful as deodorizers in the sick room or other places.

The same principle is used efficiently in another form. The vapor of wood alcohol is passed over the surfaces of asbestos containing particles of finely divided platinum. This apparatus has given very good results, and for a given amount of disinfection leaves less odor of formaldehyde gas in the room than any other. The apparatus is, however, bulky and expensive.

**Formalin by Boiling and Passing the Vapor through a Superheated Coil or Chamber.**—This system consists in heating the ordinary commercial formalin to a high temperature in an incandescent copper coil or chamber, and allowing the vapors to pass off freely. It is claimed for this method that the degree of heat necessary to break up the polymerized products formed is supplied, and thus a loss of formaldehyde is prevented. A further action of the intense heat in the copper tube on the solution is partially to convert the methyl alcohol contained in commercial formalin into formaldehyde gas by partial oxidation, thereby utilizing a part of the methyl alcohol and increasing the amount of formaldehyde. (Fig. 209.)

**Trioxymethylene or Paraform.**—This system consists in heating the solid polymer of formaldehyde (trioxymethylene).

There are several methods for doing this. They are somewhat expensive but efficient. The formaldehyde gas must be protected from burning.

**Formalin to which Glycerin has been Added.**—To the formalin is added 10 per cent. of glycerin, and the mixture is simply boiled in a suitable copper vessel, the steam and formaldehyde gas passing off by a tube. This is a very serviceable apparatus. When it is attempted to vaporize the formalin too rapidly part of it bubbles over in fluid form.

With 50 per cent. more of formalin than that used in the high temperature autoclave and heated tube or chamber methods, the results seem to be equally as good. The apparatus is very easy to use, and is not liable to get out of order.

Similar forms of apparatus are also employed, when, instead of glycerin, the formalin is mixed with an equal quantity of water. The water is for the purpose of giving additional moisture to the air, and, at the same time, like the glycerin, to prevent the change of formaldehyde into inert substances.

**From Formalin in an Open Pan.**—A very simple method, devised by Dr. R. J. Wilson, is to fill a tin pan with twelve ounces of formalin for each 1000 cubic feet and put this on an upright sheet of tin, which is cut so as to allow of the entrance of air below and yet protect the formalin in the pan from the flame. For heating put under it a small tin can filled with asbestos packing which has been soaked with wood alcohol. A still simpler method is to pour on folded sheets sixteen ounces of formalin per 1000 cubic feet and then stretch them out over lines in a room and leave for two hours. If the room is tightly sealed very fair surface disinfection will take place.

**Lime and Permanganate Method of Generating Formaldehyde Gas.**—Satisfactory results in disinfection have been obtained from the following combination of chemicals. Two ounces of a quick-slaking, coarsely granular lime (calcium oxide); 5 ounces of permanganate of potash;  $\frac{1}{2}$  gram oxalic acid; 5 ounces formaldehyde solution, 40 per cent. strength; and  $2\frac{1}{2}$  ounces of water. This is sufficient in quantity to disinfect 1000 cubic feet of space in five hours. It is used as follows: The lime and permanganate of potash are mixed together in a pan at least  $10\frac{1}{2}$  inches in diameter and  $3\frac{1}{2}$  to 4 inches in depth. Over this is poured the freshly prepared mixture of formaldehyde solution, oxalic acid, and water. A rapid evolution of gas takes place.

Another combination is: lime, 2.7 ounces; potassium permanganate, 5.5 ounces; formaldehyde solution, 7.4 ounces; water, 2.7 ounces. The technic is as follows: The lime and permanganate are mixed in a wide, deep pan as above, and the freshly prepared formaldehyde and water mixture is poured over it.

Permanganate of potash method. The following combination will also disinfect 1000 cubic feet of space in five hours: potassium permanganate, 10 ounces; formaldehyde solution, 40 per cent., 9 ounces; water, 4.5 ounces. The formaldehyde and water are mixed together and rapidly poured over the permanganate of potash. The reaction is immediate and violent. This mixture requires a deep, wide pan or a pail at least 18 inches deep. The addition of the water is believed to increase the liberation of the formaldehyde gas.

**Lime Method of Generating Formaldehyde Gas.**—To ten ounces of 40 per cent. formaldehyde solution slowly add one ounce of concentrated sulphuric acid; pour this solution onto two pounds of quicklime that has previously been cracked into small lumps and placed in a dairy pan not less than twelve inches in diameter. The liberation of a large amount of gas in a short time more than compensates for the loss by polymerization, and disinfection is effected by a quick union of the gas and organisms to be destroyed. Saturated solution of aluminum sulphate may be used instead of concentrated sulphuric acid.

**Rapid Generation of Formaldehyde Gas for Large Chambers by the Method of Dr. R. J. Wilson.**—The generator (Fig. 209) is made of ordinary iron steam pipe and can be manufactured in any pipe-cutting establishment in a very few hours. It consists of an outer steam jacket of six-inch pipe, two feet long, and capped at both ends. Through the upper cap there is a four-inch opening, with a thread, through which projects an inner chamber for formalin. This chamber consists of a four-inch pipe, twenty-two inches long, capped at the upper end and welded or capped at the lower end. The upper end of this pipe is so threaded

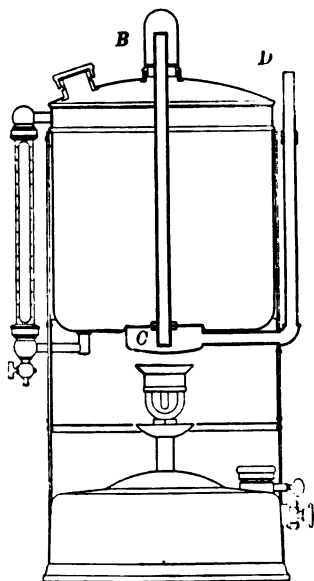


FIG. 208.—Formaldehyde apparatus.

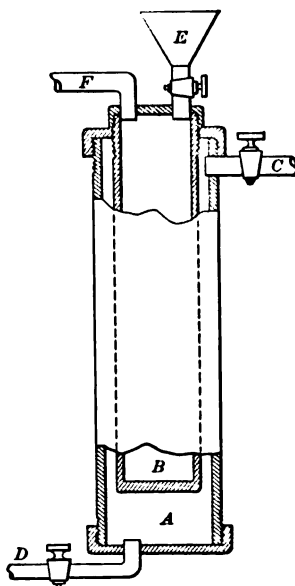


FIG. 209.—Wilson's formaldehyde generator: A, steam chamber; B, formalin chamber; C, steam supply; D, drip; E, inlet for formalin; F, outlet for formaldehyde.

as to permit of its being screwed through the cap of the steam jacket before that cap is screwed on. The cap of the formalin chamber is fitted on the same thread that passes through the cap of the steam jacket. The in-take for steam is near the top of the steam jacket, through a half-inch pipe, and the steam is controlled by a globe valve. The outlet for steam or drip is through a half-inch pipe from the bottom cap of the chamber and is also controlled by a globe valve. The intake for formalin is through the upper cap of the formalin chamber through a half-inch pipe controlled by a globe valve. The outlet for formaldehyde is a half-inch pipe through the upper cap of the formalin chamber.

As a result of the investigations undertaken in the Department of Health laboratories on methods for testing the efficiency of room disinfections, we adopted the plan of placing infected threads in the rooms. This plan adopted by us may be summarized as follows:

**METHOD FOR TESTING EFFICACY OF ROOM DISINFECTION.**—The following method modified by Dr. Schroder, working in the Research Laboratory, is now in use in the Department of Health.

The main points of the system are as follows:

No. 36 cotton is cut into inch lengths, placed in a Petri dish, and covered with a forty-eight-hour broth culture of *B. pyocyaneus*.

They are left for two or three minutes or until they are thoroughly saturated, then removed to filter-paper in another covered Petri dish and left to dry. When dry they are placed in tissue-paper envelopes, which are stamped with all necessary data. Each envelope is dated and sealed and sent to the disinfecter who places it in the room which is to be disinfected.

The driver who calls for the bedding takes up the tests, placing them in a manilla envelope and entering them upon his card. The envelopes are then returned to the laboratory where the tests and receipt card are compared and any discrepancy noted.

The test envelopes are then stamped with date of receipt, and the threads are removed and placed in a modified Ayer's medium, which is a synthetic medium and consists of the following:

Asparagin . . . . .	4
Neutral sodium phosphate . . . . .	2
Sodium lactate . . . . .	6
Sodium chlorate . . . . .	5
Water . . . . .	1000

Add enough NaOH to render the medium alkaline to litmus. This culture medium may be depended upon to give bright green color reaction in twenty-four to forty-eight hours.

The tubes are incubated for forty-eight hours and the color reaction noted and entered upon test envelope.

At the end of the week a bacteriologist's report is compiled which shows at a glance the work of each disinfecter, the number of cases of each disease for which disinfection was performed, the number of successful disinfections, the number of tests lost, etc.

**Sulphur Dioxide in House Disinfection.**—Four pounds of sulphur should be burned for every 1000 cubic feet. The sulphur should be broken into small pieces and put into a pan sufficiently large not to allow the melted sulphur to overflow. This pan is placed in a much larger pan holding a little water. The cracks of the room should be carefully pasted up and the door, after closing, also sealed. Upon the broken sulphur is poured three or four ounces of alcohol and the whole lighted by a match. The alcohol is not only for the purpose of aiding the sulphur to ignite, but also to add moisture to the air. An exposure of eight to twelve hours should be given.

Sulphur fumigation carried out as above indicated is not as efficient as formaldehyde fumigation, but suffices for surface disinfection for diphtheria and the exanthemata. All heavy goods should be removed for steam disinfection if there is any possibility of the infection having penetrated beneath their surface. If there is no place for steam disinfection their surfaces should be thoroughly exposed to fumigation and then to the air and sunlight. In many cases when cleanliness has been observed, surface disinfection of halls, bedding, and furniture may be all that will be required.



There is always a very slight possibility of a deeper penetration of infection than that believed to have occurred; it is therefore better to be more thorough than is considered necessary rather than less.

Sulphur dioxide without the addition of moisture has, as already stated under the consideration of disinfectants, very little germicidal value upon dry bacteria.

**ADVANTAGES OF FORMALDEHYDE GAS OVER SULPHUR DIOXIDE FOR DISINFECTION OF DWELLINGS.**—Formaldehyde gas is superior to sulphur dioxide as a disinfectant for dwellings; first, because it is more efficient in its action; second, because it is less injurious in its effects on household goods; third, because when necessary it can easily be supplied from a generator placed outside of the room and watched by an attendant, thus avoiding, in some cases, danger of fire.

Apart from the cost of the apparatus and the greater time involved, formaldehyde gas, generated from commercial formalin, is not much more expensive than sulphur dioxide—viz., twelve to twenty cents per 1000 cubic feet against ten cents with sulphur. Therefore, we believe that formaldehyde gas is the best disinfectant at present known for the surface disinfection of infected dwellings. For heavy goods it is far inferior in penetrative power to steam; but for the disinfection of fine wearing apparel, furs, leather, upholstery, books, and the like, which are injured by great heat, it is, when properly employed, better adapted than any other disinfectant now in use.

**Public Steam Disinfecting Chambers.**—These should be of sufficient size to receive all necessary goods, and may be either cylindrical or rectangular in shape, and are provided with steam-tight doors opening at either end, so that the goods put in at one door may be removed at the other. When large the doors are handled by convenient cranes and drawn tight by drop-forged steel eye-bolts swinging in and out of slots in the door frames. The chambers should be able to withstand a steam pressure of at least one-half an atmosphere, and should be constructed with an inside jacket, either in the form of an inner and outer shell or of a coil of pipes. This jacket is filled with steam during the entire operation, and is so used as to bring the goods in the disinfecting chamber up to the neighborhood of 220° F. before allowing the steam to pass in. This heats the goods, so that the steam does not condense on coming in contact with them. It is an advantage to displace the air in the chamber before throwing in the steam, as hot air has far less germicidal value than steam of the same temperature. To do this a vacuum pump is attached to the piping, whereby a vacuum of fifteen inches can be obtained in the chamber. The steam should be thrown into the chamber in large amount, both above and below the goods, and the excess should escape through an opening in the bottom of the chamber, so as more readily to carry off with it any air still remaining. The live steam in the chamber should be under a pressure of two or three pounds so as to increase its action.

To disinfect the goods we place them in the chamber, close tight the doors, and turn the steam into the jacket. After about ten minutes,

when the goods have become heated, a vacuum of ten to fifteen inches is produced, and then the live steam is thrown in for twenty minutes. The steam is now turned off, a vacuum is again formed, and the chamber again superheated. The goods are now thoroughly disinfected and dry. In order to test the thoroughness of any disinfection, or any new chamber, maximum thermometers are placed, some free in the chamber and others surrounded by the heaviest goods. It will be found that, even under a pressure of three pounds, live steam will require ten minutes to penetrate heavy goods.

**Practical Points on Heat Disinfection.**—In the practical application of steam for disinfecting purposes it must be remembered that while moist steam under pressure is more effective than streaming steam, it is scarcely necessary to give it the preference, in view of the fact that most known pathogenic bacteria produce no spores and the spores of the few that do develop them are quickly destroyed by the temperature of boiling water, and also that "superheated" steam is less effective than moist steam. When confined steam in pipes is "superheated" after its generation it has about the same germicidal power as hot, dry air at the same temperature. Esmarch found that anthrax spores were killed in streaming steam in four minutes, but were not killed in the same time by superheated steam at a temperature of 114° C. It should also be remembered that dry heat has but little penetrating power, and that even steam requires time to pass through heavy goods. Koch and Wolffhügel found that registering thermometers placed in the interior of folded blankets and of other large packages did not show a temperature capable of killing bacteria after three hours' exposure in a dry hot-air oven at 133° C. and over. We have put a piece of ice in the middle of several mattresses and recovered it after exposing the goods to an atmosphere of live steam for ten minutes.

**The Disinfection of Hands, Instruments, Ligatures, and Dressings for Surgical Operations.**—**Instruments.**—All instruments, except knives, after having been thoroughly cleansed, are boiled for three minutes in a 1 per cent. solution of washing soda. Knives, after having been thoroughly cleansed, are washed in sterile alcohol and wiped with sterile gauze and then put into boiling soda solution for one minute. This will not injure their edges to any great extent.

**Gauze.**—Gauze is sterilized by moist heat either in an Arnold steam sterilizer for one hour or in an autoclave for thirty minutes. It is placed in a perforated cylinder or wrapped in clean towels before putting in the sterilizer, and only opened at the operation.

Iodoform gauze is best made by sprinkling sterile iodoform on plain gauze sterilized as described above.

**Ligatures—Catgut.**—Boil for one hour in alcohol under pressure at about 97° C. It is often put in sealed glass tubes, which are boiled under pressure. These remain indefinitely sterile. The alcohol does not injure the catgut. If desired, the catgut can be washed in ether and then soaked a short time in bichloride before heating in alcohol. Böckman, of St. Paul, suggested wrapping the separate strands of

catgut in paraffin paper and then heating for three hours at 140° C. This procedure prevents the drying out of the moisture and fat from the catgut, so that it remains unshrivelled and flexible after its exposure. Darling, of Boston, tested this method and found it satisfactory. Dry formaldehyde gas does not penetrate sufficiently, and is not reliable. Silver wire, silk, silkworm gut, rubber tubing, and catheters are boiled the same as the instruments.

**Hand-brushes.**—These should be boiled in soda solution for ten minutes.

**The Skin of the Patient.**—It is impossible absolutely to sterilize the deeper portions of the skin, but sufficient bacteria can be removed to render infection rare. The skin is washed thoroughly with warm green soap solution, then with alcohol, and finally with 1 to 1000 bichloride. A compress wet with a 25 per cent. solution of green soap is now placed on, covered with rubber tissue, and left for three to twelve hours; and after its removal the skin is washed with ether, alcohol, and bichloride solution, and then covered with a gauze compress previously moistened with a 1 to 1000 bichloride of mercury solution. At the operation the skin is again scrubbed with green soap solution followed by ether, alcohol, and then with the bichloride of mercury solution. In some places the bichloride compress is replaced one hour before the operation by a pad wet in 10 per cent. solution of formalin.

**The Hands.**—Fürbinger's method, slightly modified, is now much used, and gives good results. The hands are washed in hot soap and water for five minutes, using the nail-brush. They are then soaked in 85 per cent. alcohol for one minute and scrubbed with a sterile brush. They are finally soaked in a 1 to 1000 bichloride of mercury solution for two minutes. The alcohol and bichloride of mercury are sometimes combined and used together. Another method which gives good results is as follows: Skin of operator is scrubbed for five minutes with green soap and brush, then washed in chlorinated lime and carbonate of soda in proportions to make a good lather; washed off in sterile water, and then scrubbed with brush in warm bichloride solution 1 to 1000.

Owing to the risk of leaving untouched bacteria under the nails and in cracks of the skin, sterilized rubber gloves are now being used more and more in operations. Some surgeons prefer sterilized cotton gloves frequently changed. The gloves can be sterilized by steam.

**Mucous Membranes.**—Here absolute sterilization cannot be achieved without serious injury to the tissues. Those of the mouth and throat are cleansed by a solution consisting of equal parts of peroxide of hydrogen and lime-water. In the nostrils it is better to employ the milder solutions, such as diluted Dobell's or Listerine. These are also used in the mouth instead of the peroxide. Wadsworth urges the use of preparations containing about 30 per cent. of alcohol as being very efficient. Dakin's solution (see below) may also be tried.

The vagina is swabbed out thoroughly with sterile warm soap and water, and then irrigated with a 2 per cent. carbolic acid or a 1 to 1000 bichloride of mercury solution.

**Disinfection of Wounds.**—The immense number of wounded in the European War has led to a further study of the use of antiseptic solutions.

The great efficiency of chlorine and some of its less stable compounds as purifying, deodorizing and bleaching agents has for a long time been known. Its action was attributed to the strong indirect oxidizing influence exerted by it. Chlorine gas and calcium hypochlorite, "bleaching powder" or "chloride of lime" were recognized as among the most potent, though destructive disinfectants. Potassium and sodium hypochlorite were also employed as disinfectants and to some extent as antiseptics, though the latter use was restricted by the irritating action of these substances.

It was not until the present war in Europe created a demand for more efficient antiseptics than those in general surgical use that recourse was made to chlorine compounds in which the chlorine existed in a combination from which it could be readily liberated in active form according to requirements.

The researches of Dr. H. D. Dakin, at first associated with Dr. Alexis Carel in France and subsequently commissioned by the British Government, demonstrated that sodium hypochlorite in strictly neutral solution and not exceeding 0.5 per cent. in concentration, could be used freely in surgical practice without occasioning discomfort.<sup>1</sup> His studies on the action of this antiseptic showed that when it acted on protein substances it conferred antiseptic properties on them and that the chlorine presumably became linked to a nitrogen of one or more of the amino acids composing the protein. This led to the inference that soluble substances possessing a nitrogen-chlorine linkage would be likely to have antiseptic properties. This was verified by experiment. The group of substances, called chloramines, which have this linkage, contain many preparations which are soluble and very slightly, if at all, toxic. The cheapest of these, because it can be manufactured from a waste substance obtained in the production of saccharin, is paratoluene-sodiumsulphochloramide,  $C_6H_4SO_2Na.NCl.3H_2O$ . This substance is obtained in white crystals, readily soluble in water and singularly stable both dry and when in solution. It readily parts with chlorine when

<sup>1</sup> A neutral sodium hypochlorite solution of proper strength, "Dakin's solution," can be prepared as follows: Dissolve 14 grams of dry sodium carbonate (or the equivalent of the hydrated crystals) in 1 liter of water, add 20 grams of fresh bleaching powder with not less than 30 per cent. available chlorine and shake the mixture vigorously for at least five minutes. Allow it to stand for half an hour and then filter. To the clear filtrate, add 2 grams of boric acid, which should so completely neutralize the solution that a particle of solid phenolphthalein will not become pink when moistened with it. It is also possible to prepare a neutral solution of sodium hypochlorite without the addition of boric acid, if sodium bicarbonate is employed. With the bleaching powder of the quality mentioned, the proportions may be chosen as follows: 45 grams dry sodium carbonate and 45 grams of sodium bicarbonate are dissolved in 1 liter of water. One hundred grams of bleaching powder are well shaken with 1 liter of water and then the alkaline solution added and the whole again thoroughly shaken. The precipitate is then removed by filtration. The resulting clear solution should react neutral to phenolphthalein. Should this not be the case, a little carbon dioxide may be passed into the solution to neutralize the residual trace of acid. This solution is three times the strength which should be used. When required, it should be diluted with two volumes of water. Sodium hypochlorite solutions deteriorate. They should not be used when over a week old.

brought into contact with easily oxidizable substances, and is a powerful antiseptic. Proteins are not precipitated by this chloramine, and its penetration when applied to the tissues is unusually great. As it is decomposed in exerting its action, it should be frequently renewed, but its non-toxic and unirritating characters permit its free and abundant use. Besides Dakin's solution other antiseptic irrigation fluids are being experimented with in the treatment of the wounded, and also ointments and powders. It is believed that for certain infections special antiseptics should be selected.

**Hypodermic and Other Syringes.**—These when not boiled are sterilized by drawing up into them boiling water a number of times and then finally a 5 per cent. solution of carbolic acid, the acid after three minutes to be washed out by boiling water. If cold water is used the carbolic solution should remain in the barrel for ten minutes. Great care should be taken to wash out all possible organic matter before using the carbolic acid or boiling to sterilize. Syringes made entirely of glass or of glass and asbestos can be boiled in soda solution.

**The Sterilization of Milk.**—Complete sterilization destroys all the germs in milk, and so, if no new ones gain entrance, prevents permanently fermentative changes. This requires boiling for fifteen to forty-five minutes on two or three consecutive days, according to the presence or absence of certain spores.

Milk is best sterilized by heat, for nearly all chemicals, such as boric acid, salicylic acid, and formalin, are not only slightly deleterious themselves but also make the milk less digestible, and therefore less fit for food. Formalin is the least objectionable of the three. Milk may be sterilized at a high or low temperature—that is, at the boiling temperature—or at a lower degree of heat, obtained by modifying the steaming process. Milk heated at as high a temperature as 100° C. is not altogether desirable for prolonged use for infants, as the high temperature causes certain changes in the milk which make it less suitable as a food for them.

**Pasteurization.**—These changes are almost altogether avoided if a temperature below 70° C. is used. It is recommended, therefore, that the lowest temperature be used for partial sterilization which will keep the milk wholesome for twenty-four hours in the warmest weather and kill the tubercle, typhoid, and other non-spore-bearing bacilli. Raising the milk to a temperature of 60° C. for twenty minutes, 65° C. for fifteen, 70° for five, 75° for two, or 80° for one will accomplish this. Exposure for even one minute at 70° destroys 98 per cent. of the bacteria which are not in the spore form. Fully 99 per cent. of tubercle bacilli are destroyed. This subject is considered more fully in the chapter on Milk. One of the many forms of apparatus is the following:

(a) A tin pail or pot, about ten inches deep by nine inches in diameter, provided with the ordinary tin cover which has been perforated with eight holes each an inch in diameter.

(b) A wire basket, with eight nursing bottles (as sold for this purpose in the shops).

(c) Rubber stoppers for bottles and a bristle brush for cleaning the bottles.

**Directions** (Koplik).—Place the milk, pure or diluted (as the physician may direct), in the nursing bottles and place the latter in the wire basket. Put only sufficient milk for one nursing in each bottle. Do not cork the bottles at first.

Having previously poured about two inches of water in the tin pail or pot and brought it to the boiling-point, lower the basket of nursing bottles slowly into the pot. Do not allow the bottles to touch the water or they will crack. Put on the perforated cover and let the steaming continue for ten minutes; then remove the cover and firmly cork each bottle. After replacing the cover, allow the steaming to continue for fifteen minutes. The steam must be allowed to escape freely or the temperature will rise too high.

The process is now complete. Place the basket of bottles in a cool, dark place or in an ice-chest. The bottles must not be opened until just before the milk is to be used, and then it may be warmed by plunging the bottle in warm water. If properly prepared the milk will taste but little like boiled milk.

The temperature attained under the conditions stated above will not exceed in extreme cases 87° C. (188° F.).

A different but admirable method is the one devised by Dr. Freeman. Here a pail is filled to a certain mark with water, and then placed on the stove until the water boils. It is then removed, and immediately a milk-holder, consisting of a series of zinc cylinders, is lowered with its milk bottles partially full of milk. The cover is again applied. The heat of the outside water raises the temperature of the milk in ten minutes to about 65° C. (150° F.), and holds it nearly at that point for some time. After twenty minutes the milk is removed, placed in cold water, and quickly cooled. The milk is kept in the ice-chest until used. When milk is pasteurized in great quantities it should always be done by the "holding process," as "flash" pasteurization is unreliable. Milk should be pasteurized when it is as fresh as possible, and only sufficient milk for twenty-four hours should be pasteurized at one time. If after nursing the infant leaves some milk in the bottle this should be thrown away.

**Care of the Bottles.**—After nursing, the bottles should be filled with a strong solution of washing soda, allowed to stand twenty-four hours, and then carefully cleaned with a bristle (bottle) brush. The rubber stoppers and nipples after using should be boiled in strong soda solution for fifteen minutes and then rinsed and dried.

After sterilizing, milk should never be put into unsterilized bottles, as this will spoil it.

### GENERAL CONCLUSIONS ON DISINFECTION.

The previous pages have shown that it is comparatively easy to destroy microbes by germicides or heat when access to them is attainable. Foods, instruments, clothing, bedding, the excreta, the surface

of the body, etc., can be readily disinfected, but when we try to disinfect the mucous membranes of the living person we fail.

*The Importance of Disinfection of Surroundings after Recovery or Death from Infectious Diseases.*—Year by year knowledge is accumulating which indicates that nearly all cases of spread of infection are due to the immediate transfer from a living carrier to the person who contracted the disease. The carrier may be diseased or may simply harbor the germs. If cleanliness is maintained throughout the disease there is, as a rule, little need of specific disinfection after recovery.

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